Japanese Encephalitis Virus Infects Neuronal Cells through a Clathrin Independent Endocytic Mechanism

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Running Title: Clathrin independent endocytosis of JEV

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Word Count: Abstract = 180; Text = 6574
Abstract

Japanese encephalitis virus (JEV) is a mosquito-borne pathogenic flavivirus responsible for acute viral encephalitis in humans. The cellular entry of JEV is poorly characterized in terms of molecular requirements and pathways. Here we present a systematic study of the internalization mechanism of JEV in fibroblasts and neuroblastoma cells. To verify the role of distinct pathways of cell entry, we used fluorescently labelled virus particles, a combination of pharmacological inhibitors, RNA interference (RNAi) and dominant negative (dn) mutants of regulatory proteins involved in endocytosis. Our study demonstrates that JEV infects fibroblasts in a clathrin dependent manner, but it deploys a clathrin independent mechanism to infect neuronal cells. The clathrin independent pathway requires dynamin and plasma membrane cholesterol. Virus binding to neuronal cells leads to rapid actin rearrangements, and an intact and dynamic actin cytoskeleton, and the small GTPase RhoA plays an important role in viral entry. Immunofluorescence analysis of viral colocalization with endocytic markers showed that JEV trafficks through Rab5 positive early endosomes and release of the viral nucleocapsid occurs at the level of the early and not the late endosomes.
**Introduction**

Japanese encephalitis virus (JEV) belongs to the genus *Flavivirus* in the family *Flaviviridae*. Other members of the genus include Dengue virus (DENV), West Nile virus (WNV), Yellow fever virus (YFV), and Tick-borne encephalitis virus (TBEV). Most flaviruses are transmitted by mosquito or tick vectors and cause serious human and animal disease (46). JEV is a major cause of epidemic encephalitis worldwide, with a potential to cause permanent neuropsychiatric sequelae and is sometimes fatal in children living in endemic areas such as Southeast Asia. JEV transmission has also been observed in the Southern Hemisphere and has the potential to become a worldwide public health threat (49, 69).

JEV particles are small (~50 nm), in which a glycoprotein-containing lipid envelope surrounds the capsid, which has a single-stranded positive-sense 11-kb RNA genome. The viral RNA encodes a single open reading frame carrying genes for three structural proteins—capsid (C), premembrane (prM) and envelope (E), and seven non-structural (NS) proteins—NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5 (46). The E glycoprotein is the major antigenic determinant on flavivirus particles, which mediates binding and fusion during virus entry (7, 41). After internalization, flaviviruses are trafficked to an endosomal compartment where low pH induces conformational changes necessary for virus uncoating and capsid disassembly (31).

Clathrin mediated endocytosis (CME) is believed to be the major route of flavivirus cell entry. The earliest evidence for this was obtained from ultrastructural studies showing the presence of Kunjin and YFV in coated pits (32, 63). Subsequently DENV and WNV have also been shown to infect mosquito cells via CME (3, 18, 55). Single particle tracking of dengue virus in live cells has demonstrated virus movement along the cell surface in a
diffusive manner before it is captured by a pre-existing clathrin coated pit (80). Till date only one study has reported an alternate clathrin independent infectious entry pathway for DENV-2 in mammalian cells (1). Using biochemical inhibitors and dominant negative mutants, entry of DENV-1 was demonstrated to be clathrin dependent, while that of DENV-2 was clathrin, cholesterol and caveolin independent in Vero cells. In contrast, DENV-2 entry in A549 cells was clathrin dependent, as previously reported for HeLa, C6/36 and BS-C-1 cells (2). It is possible that the entry pathway(s) utilized by the virus maybe cell type dependent. Two recent RNA interference studies for Dengue virus entry and multiplication into monocytes and HepG2 cells show dependence on clathrin heavy chain and dyamin-2 (5, 6).

The cell biology of JEV entry remains relatively unexplored. An electron-microscopy study showed that JEV is transported across cerebral blood vessels and breaches the blood brain barrier, and both coated and uncoated vesicles could be seen in the capillary endothelium (47). Data in support of a clathrin mediated pathway for JEV internalization is limited mainly to pharmacological studies using the inhibitor chlorpromazine (62) and use of dominant negative constructs of Eps15, a key protein involved in CME (24). It has also been shown that Bafilomycin, a specific inhibitor of vaculoar type H+- ATPase, interfered with JEV infection of Vero cells (61).

In addition to CME, several endocytic pathways that do not use clathrin have also been described (43, 66). At least three clathrin-independent internalization pathways have been reported in mammalian cells, but they are not yet completely characterized (39, 52). These pathways vary in cargoes they transport and in the protein machinery that facilitates the endocytic process. Some of these pathways are constitutive, whereas others are triggered by specific signals or are hijacked by viruses (50). Considerable plasticity exists in these endocytic mechanisms and certain components such as dynamin-2, Rho GTPases and actin can participate in more than one pathway. The large GTPase dynamin-2 was originally noted...
for its role in severing clathrin-coated vesicles from the plasma membrane and was subsequently found to be involved in a clathrin independent pathway mediated by caveolae (66). In addition, some members of the ADP-ribosylation factor (Arf) and Rho subfamilies of small GTPases were recently suggested to play key roles in regulating clathrin independent endocytic pathways (58). Rho GTPases are also known to be involved in the control of actin dynamics (33). Dynamin-2 and F-actin are crucial to most endocytic processes that coexist within the cell. These common factors must be tightly controlled and perhaps differentially regulated according to the endocytic mechanism.

It is now being increasingly appreciated that many viruses can utilize more than one entry pathway to infect cells (34, 50). There have been many recent studies showing that viruses can induce macropinocytosis for productive entry and infection (25, 54, 71). Here, we address the role of different endocytic molecules and pathways involved in JEV internalization in Vero (green monkey kidney fibroblasts), Neuro2a (mouse neuroblastoma) and SH-SY5Y (human neuroblastoma) cells. Using a combination of pharmacological and molecular approaches, we show that a clathrin independent pathway operates in neuronal cells for JEV infection. Dynamin-2, membrane cholesterol and a dynamic actin cytoskeleton are specifically required for neuronal cell entry and infection of JEV. In addition, the small GTPase Rho A and myosin II motors aid JEV internalization. Immunofluorescence analysis of viral colocalization with endocytic markers showed that JEV is trafficked to Rab5 positive early endosomes where membrane fusion occurs. The infection process in both fibroblasts and neuronal cells requires acid dependent fusion at the level of the early endosomes.

Materials and Methods

Cells, antibodies, inhibitors and plasmids
Mouse neuroblastoma (Neuro2a) and human neuroblastoma (SH-SY5Y) cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (HyClone). Porcine stable kidney (PS) cells (National Center for Cell Sciences, Pune, India) and Vero (African green monkey kidney) cells were grown in Eagle’s minimal essential medium (MEM) with 10% FBS. All media was additionally supplemented with 100µg/ml penicillin/streptomycin and 2mM L-glutamine. Antibodies to JEV-E, actin (loading control) and myc were from Abcam. CHC antibody was from Cell Signaling Technology, and CLC antibody from Santa Cruz. All inhibitors- chlorpromazine hydrochloride, dynasore, EIPA (5-N-ethyl-N-isoproamiloride), methyl-β-cyclodextrin, filipin, cytochalasin D, latrunculin A, jasplakinolide, blebbistatin and bafilomycin were purchased from Sigma. The chemical inhibitor for Rho A (CT04) and the Rho, Rac, Cdc42 G-LISA Activation Assay Biochem kits were purchased from Cytoskeleton Inc (Denver, CO). All chemical inhibitor stock solutions were prepared according to manufacturer’s directions.

Fluorescent dye- DiD, fluorophore coupled- transferrin, - phalloidin, - secondary anti-mouse and anti-rabbit antibodies, and ProLong Gold anti-fade reagent with DAPI, were obtained from Invitrogen Corporation. HRP coupled secondary antibodies were obtained from Jackson Immunochemicals. The Eps15 mutants: DIIIδ2(control), DIII all subcloned in pEGFP-C2 were kind gifts from Alexandre Benmerah (Universite Paris, Paris, France) (9). Plasmids for wild type (wt), dominant active (DA), dominant negative (DN)- Rho, Rac, Cdc42, Rab5, Rab7, and dyn-2K44A were obtained from Addgene. The details are as follows- plasmids- 15899, 15900, 15901, 15903, 15904, 15905, 15906, 15907 deposited by Alan Hall (64); plasmids- 12660, 12605 deposited by Richard Pagano (17); plasmid- 14437 deposited by Ari Helenius (82); plasmid -28045 deposited by Qing Zhong(78); plasmid-34687 (deposited by Sandra Schmid).

Virus generation, purification and fluorescent labelling
For all studies JEV isolate Vellore P20778 generated in PS cells was used. The culture supernatant was harvested when 75% of the cells showed cytopathic effect, usually 36-48 h after infection, and clarified by centrifugation at 1000xg for 30 min at 4°C. Virus titres were determined using monolayers of PS cells as described earlier (83). Virus was further purified over a 20% sucrose cushion in a Beckman Coulter ultracentrifuge (Optima L-100K) at 80,000 x g for 4 hours at 4°C. Purified virus was exchanged into PBS through cycles of concentration by centrifugation (800 xg) and dilution with PBS, using 50ml ultrafiltration tubes (10kD, Amicon). Virus was labelled with DiD by injecting 2 nmol of dye into virus stock under intensive vortexing for 10 min at RT. Excess dye was removed by purification through Micro Spin™ G-25 columns (GE Healthcare). Labelling did not abolish viral infectivity. Labelled virus was used immediately for experiments.

Cell transfection and transient expression

Cells were transfected with Lipofectamine 2000 (Invitrogen) according to manufacturers’ protocols. For transfection, cells were grown to 60-70% confluency on 18mm coverslips or 35mm coverslip dishes and transfected with 1µg DNA. Transfections were typically allowed to proceed for 18-24 h before infection with JEV.

siRNA depletion experiments

Mouse specific clathrin heavy chain (CHC) targeting shRNA plasmid DNAs (SHCLND_NM_001003908) were purchased from Sigma. Best silencing efficiency was observed with Clone NM_001003908.1-5688s1c1 containing the sequence-CCGGGCCGACAAAGACAACACTAATCTCGAGATTAGTGTTGTCTTTGTCGGCTTT TTG. Cells were transfected with shRNA plasmid and harvested at 24, 48, and 72 h to check for protein knock-down. Significant depletion of CHC was observed at 72 h. For clathrin light chain (CLC) depletion a shRNA construct was generated by cloning appropriate
oligonucleotides containing the targeting sequence in the pSIREN-RetoQZsGreen plasmid (Clontech). The sequence of the forward primer oligonucleotides designed for the CLC shRNA was GATCCGGAGCCTGAAAGTATCCGTATTCAAGAGATACGGATACTTTTC AGGCTCTTTTTTGCAGC. This sequence of the clathrin light chain targeted in the shRNA construct is evolutionarily conserved from rodents to primates. The GFP-CLCshRNA clone was used for gene silencing in both Neuro2a and Vero cells. This construct confers the additional advantage that cells receiving the shRNA also express GFP. A time course of CLC depletion was done and maximal protein knock-down was observed at 72 h. Cell lysates were run on SDS-PAGE and western blotting was done for CLC, CHC and actin. For infection experiments, cells were transfected with shRNA plasmids for either CHC or CLC for 72 h following which they were infected with JEV.

**Virus infection and cell treatment**

Neuro2a cells seeded on 35mm coverslip dishes or 18mm coverslips were either transfected as described above or treated with inhibitors prior to infection. The incubation time, dosage and cell viability upon treatment was determined for each inhibitor. For all inhibitors the final DMSO concentration never exceeded more than 0.2% of the total culture medium. Cells were infected with JEV at MOI 0.4 for 1 h at 37°C with or without inhibitors. For experiments involving dynasore, cells were grown in 10% Nuserum (BD Biosciences) containing media. For cholesterol depletion cells were washed four to five times with serum free medium before addition of methyl-β-cyclodextrin (MβCD) or filipin. For Rho A inhibition, cells were serum starved for 2 hours, and inhibitor-CT04 added to serum free media for 2 hours. For inhibitors requiring serum free conditions infections were done using purified virus in PBS with 0.1% glucose. For experiments involving transfections cells were infected with JEV at MOI 1 (results in about 30% infection in GFP transfected cells) for 1h at 37°C. Following infection, cells were washed twice with PBS and complete media was added. Cells were fixed 24 hours
post-infection (hpi) in 2% paraformaldehyde and permeabilised using 0.4% TritonX-100 or 0.04% saponin in PBS for 20 min at RT. Blocking was done with 2mg/ml BSA in PBS for 1 h prior to incubation with anti-JEV E antibody, followed by Alexa488/568 anti-mouse secondary antibody. For transfection experiments using over-expression constructs of myc-tagged Rho, Rac and Cdc42, cells were processed for double immunostaining with antibodies against Myc epitope tag and JEV E antigen.

**Virus colocalization studies**

For transferrin and JEV colocalization studies, labelled virus (MOI 10) was allowed to bind cells on ice for 1h. After virus binding, cells were incubated with labelled Tf for 5, 10, and, 15 min at 37°C. For colocalization with dsRed-Rab5, and GFP-Rab7, labelled virus binding to cells was done on ice for 1h, after which cells were warmed to 37°C for the specified time points. Cells were given a low pH wash (0.1M sodium acetate, 0.05M NaCl, pH5.5, 5min) to remove cell sticking virus particles, fixed in 2% paraformaldehyde and imaged.

**Immunofluorescence microscopy and image processing**

Confocal microscopy was performed using an Olympus FV1000 confocal microscope. For quantification of entry, images were acquired with 20X Plan Apo objective, NA 1.20. Images were acquired for 8-10 fields of view per coverslip. Quantification of JEV infection was done by counting cells that were immune-stained versus non-stained for virus envelope (E) antigen. For experiments involving transfections, quantitation was done by counting cells that were both transfected and infected versus those that were transfected but remained uninfected. The infection studies were normalized to either solvent treated or GFP expressing controls. All experiments were done in duplicate or triplicate. Results are expressed as mean ± SD. Significance was determined using a Student T-test. For colocalization experiments, images were acquired with a 60X PlanApo objective lens (NA 1.4). Z-stacks were acquired at
0.25µm per slice by sequential scanning. FluoView software (Olympus) was used to generate
cross-sectional and maximum intensity projection images.

**Quantitative real-time (qRT) PCR**

Neuro2a cells were plated in 35mm dishes at a density of 0.5x10^6 cells/ dish and were pre-
treated with inhibitors. Viral adsorption (MOI 10) to cells was performed at 4°C for 1h,
followed by one wash with cold PBS, and a shift to 37°C for 1h in the presence of inhibitor.
Post incubation, cells were washed with chilled PBS and low pH buffer and lysed in Trizol
reagent (Invitrogen). qRT-PCR primers were procured from Sigma. JEV positive strand
cDNA was generated using the following primer- AATAAGTTGTAGTTGGGGCAGCTCTG.
JEV was amplified using the following probes- Taqman probe:
CCACGACTACCGACCCATAGACTG (5’ end FAM, 3’end TAMRA), 5’ primer:
AGACACACAGGAATGAGATG, 3’ primer:
AATAAGTTGTAGTTGGGGCAGCTCTG. GAPDH was used as an internal control- Taqman
probe sequence: ACAACCTGGTCCTCAGTGC (5’ end FAM, 3’end TAMRA), 5’
primer: CCTGCCAAGTATGACTGAC, 3’ primer: GGAGTTGCTGTGATCG. The PCR
conditions were as follows: 94°C for 2min (1 cycle), 94°C for 15 sec, 55°C for 30sec and
72°C for 1min (40 cycles). qPCR was done on Applied Biosystems ABI 7500.

**Quantification of Transferrin uptake by flow cytometry and microscopy**

Transferrin internalization by cells after treatment with chlorpromazine was measured by
flow cytometry. Cells pre-treated with 25µM or 50µM chlorpromazine were given a 10 min
pulse of Alexa488-Tf in labelling medium (DMEM or MEM 10% serum). After incubation,
excess label was washed off with chilled PBS and low pH buffer to remove surface bound
Tf. Cells were fixed, detached and Alexa488 fluorescence was analysed using a Becton
Dickinson (BD) FACSCantoII flow cytometer. The average of measured geometric means of
internalized Tf in control and inhibitor treated cells was calculated. For quantification of transferrin uptake in cells transfected with different endocytic mutants/CLC shRNA, a pulse of Alexa568/647 Tf was given for 10 min in labelling medium. Cells were processed as described above, fixed and imaged at 20X. Total fluorescence intensity was calculated per cell using Olympus FV1000 analysis software. In each experiment fluorescence was calculated from 10-12 fields of view from duplicate slides for each transfection condition. Integrated values of cell fluorescence were corrected for background autofluorescence. Tf uptake is represented as Mean and standard error of the mean of integrated fluorescence intensity from two independent experiments.

**Rho GTPase activation assays**

Neuro2a cells were transfected with Wt, DA, DN plasmids of Rho, Rac and Cdc42. Rho, and Cdc42 activation was measured 24h post-transfection with G-LISA activation kit (Kit # BK124, Kit # BK 127 Cytoskeleton Inc.). To measure Rac activation, mock, Rac DA and Rac DN transfected cells were serum starved for 24h, followed by addition of complete media for 10min before washing with cold PBS and lysis (Kit # BK125). To measure Rho activation in response to virus binding, JEV (10MOI) was adsorbed to cells at 4°C for 1h, followed by one wash with cold PBS, and a shift to 37°C to allow infection until indicated times. Cells were washed with cold PBS, lysed and processed for ELISA. The activated form of the G-protein was detected by incubation with specific primary antibody followed by a secondary antibody conjugated to HRP and a detection reagent. The signal was read by measuring absorbance at 490nm using a microplate reader.

**Results**

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JEV internalization is dynamin-2 dependent

Dynamin-2 is a large GTPase which acts by mediating release of newly-formed endocytic vesicles from the plasma membrane. It thus plays a critical role in CME as well as some of the non-clathrin dependent pathways (26). To examine the role of dynamin-2 in JEV entry in different cell lines, the effect of dynasore, a potent and specific dynamin-2 inhibitor was tested (48). Neuro2a, SH-SY5Y and Vero cells treated with 80µM dynasore showed close to 90% block in JEV infection (Figure 1A, C, D). At 80µM, dynasore specifically blocks dynamin-2 function, which was confirmed via a significant inhibition in internalization of transferrin (Tf), a cargo that gets internalized via the dynamin and clathrin dependent endocytic mechanism. To confirm that dynasore is blocking JEV endocytosis and not inhibiting any downstream event necessary for infection, qRT-PCR of JEV positive strand RNA was performed 1hpi to estimate viral entry in untreated and dynasore treated Neuro2a cells. There was a 70% decrease in the viral load in dynasore treated cells relative to control (Figure 1B), implying that JEV endocytosis is likely to require functional dynamin.

We next tested the ability of a plasmid expressing the dominant-negative- K44A mutant of dynamin-2 (dyn2K44A (22)) to block JEV infection. Neuro2a cells were transfected with either control GFP plasmid (Figure 1E, left panel) or GFPdyn2K44A (Figure 1E, right panel) and 24 h later infected with JEV. Infected cells were immunostained for JEV-E protein. Whereas, cells expressing GFP alone were infected with JEV (Figure 1E, left panel), those expressing the dyn2K44A mutant showed nearly a 70% block in infection (Figure 1E right panel & quantitation in Figure 1F). This observation was also confirmed in SH-SY5Y and Vero cells (Figure 1F). These experiments thus suggest that JEV internalization in cells is dynamin-2 dependent.

JEV internalization is independent of clathrin mediated endocytic cargo
Since JEV infection was dynamin dependent, we examined the role of clathrin in the internalization process. Transferrin, which binds to the transferrin receptor, is a well characterized cargo of clathrin coated pits, and served as a control in these studies.

As a first approach we used fluorescently labelled virus particles to follow endocytic internalization of JEV. To detect single JEV particle entry events in Neuro2a cells, we labelled virions with membrane-permeable lipophilic dye DiD. This approach has been used successfully with Dengue, Influenza and Hepatitis C virus (21, 42, 80). A homogeneously labelled particle suspension was obtained as indicated by confocal microscopy of labelled particles attached to glass coverslips (Figure 2A, left panel). A majority of the DiD-labelled viral particles have uniform fluorescence emissions. To confirm that the DiD signal is specific to labelled JEV particles, we immuno-stained DiD-JEV with JEV-envelope antibody (Figure 2A, middle and left panel, insets). There was no significant loss in infectivity of DiD labelled virus particles as tested by plaque assays (Figure 2B).

DiD-JEV was allowed to bind to Neuro2a cells on ice for 1 h followed by pulse with labelled Tf for different time points. By 10 min virus particles can be seen associated with the cell body with several virus particles attached to filopodia (Figure 2C, upper right panel, arrowheads). At this time point no co-localization of the internalized virus particles is seen with Tf (Fig 2C, upper left panel, arrowheads). Virus internalization was a slow process and the virus containing vesicles remained segregated from Tf endosomes till 15 min post entry. (Figure 2B, lower left panel, arrowheads). After 30 min of internalization there was about 50% co-localization between virus and Tf endosomes and could this could imply presence of the virus in Rab5 early endosomes (Fig 2B, lower right panel, arrows). Virus entry in Neuro2a cells appeared to be independent of Tf endocytosis, since most of the internalized virus was seen in vesicles that did not co-localize with Tf endosomes at early time points.
JEV entry in Neuro2a cells is clathrin independent

The role of clathrin mediated endocytosis in JEV infection was further tested by employing different strategies to disrupt the pathway. Chlorpromazine is commonly used to inhibit clathrin-mediated endocytosis, however, this drug also elicits other secondary effects on the cell, including inhibition of voltage gated potassium channels in neuronal cells, and inhibition of lysosomal and cytosolic phospholipases (51, 72). Studies have shown that chlorpromazine can inhibit JEV infection of Vero cells (62). We tested this effect of chlorpromazine on Neuro2a, SH-SY5Y and Vero cells. A concentration of 50µM was chosen for infection studies as this dosage showed maximum (nearly 50% or more for Neuro2a, SH-SY5Y and 35% for Vero) inhibition in Tf uptake in all three cell lines (Figure 3A), and exposure to higher drug concentrations lead to significant cell death. While there was greater than 60% block in JEV infection of Vero cells, there was no significant inhibition of infection of Neuro2a and SH-SY5Y cells (Figure 3B, D). Treatment of Neuro2a cells with chlorpromazine also did not have any effect on the amount of JEV endocytosed as calculated by qRT-PCR (Figure 3C). This data suggests that JEV entry is likely to be cell type dependent.

The role of clathrin mediated endocytosis was further tested by the expression of GFP tagged dominant negative (dn) Eps15 mutants. Eps15 is a crucial component of clathrin-coated pits where it interacts with adaptor protein (AP)-2, the major clathrin adaptor complex. Clathrin mediated endocytosis can be blocked by overexpression of the dn Eps15 mutant- DIII, which has a large N-terminal deletion leaving only its C-terminal DIII domain intact (9). The DIII mutant with an additional deletion of its AP-2 binding sites (DIIIδ2), was used as a control to monitor over-expression effects. Overexpression of the dn Eps15 decreased transferrin internalization in Neuro2a cells by around 50% (Figure 4A vs 4B, quantitation in Figure 4C). However, when cells over-expressing the dn Eps15 mutant and
control plasmids were infected with JEV, there was a significant block in infection in Vero cells whereas Neuro2a and SH-SY5Y showed infection comparable to control (Figure 4D, E). These results imply that JEV internalization in neuronal cells can occur independently of the clathrin-mediated endocytic pathway.

To further dissect the clathrin independent endocytic mechanism in neuronal cells we focussed on the cell line Neuro2a along with Vero fibroblasts. The main components of clathrin-coated pits and vesicles are clathrin triskelions, consisting of three heavy and three light chains. To specifically inhibit clathrin mediated endocytosis, we used siRNA to knockdown the expression of both clathrin light chain (CLC) and clathrin heavy chain (CHC) in independent experiments. Both siRNAs have been previously used to inhibit clathrin-mediated endocytosis (56). CLC was depleted by use of a GFP-tagged shRNA construct that targets a evolutionarily conserved region of the gene. Over-expression of this construct in Neuro2a cells showed a significant inhibition (~60%) of Tf internalization in GFP positive cells by 72 hours (Figure 5A, compare left panel -mock transfected vs right panel-CLC shRNA transfected, quantitation in Figure 5B) and nearly 90% of CLC was depleted from cells as tested by Western blotting in both Neuro2a and Vero cells (Figure 5C). Neuro2a cells depleted of CLC did not show any reduction in JEV infection, compared to cells transfected with shRNA vector alone, whereas Vero cells showed close to 50% inhibition of infection (Figure 5D, E). Depletion of CHC in Neuro2a cells (Figure 5F) also did not show any significant decrease in JEV infection compared to mock transfected cells (Figure 5G, H). These experiments strongly suggest that JEV internalization in mouse neuroblastoma cells can occur efficiently via a clathrin independent mechanism.

Cholesterol is required for JEV infection
Since a number of viral entry pathways depend on cholesterol, we next examined whether JEV internalization was sensitive to lowering membrane cholesterol levels, which is a major determinant of many endocytic processes (65). Though several studies imply the requirement of cholesterol to be an indicator of caveolar/lipid-raft endocytosis, cholesterol-dependence of cell entry is an operational definition and does not imply a specific pathway of entry (52). Most endocytic pathways are sensitive to cholesterol perturbation, with both clathrin-dependent (70, 77) and clathrin-independent pathways (15, 16, 58) being inhibited by removal of cholesterol. Also cargo like the virus SV40 and cholera toxin B that were earlier believed to be markers for caveolar endocytosis, can be internalized via caveolin independent pathways (23, 38). Thus the definition of a caveolar endocytic pathway in terms of its specific cargo is now redundant.

For the Dengue and West Nile flaviviruses, cholesterol was shown to be required for infection (44, 53, 68). Mouse neural stem/progenitor cells depleted of cholesterol before JEV infection also showed a reduction in viral load and infective virus particle production (24). In contrast, another study showed an increase in JEV infection upon treatment of Huh-7 cells with methyl-β-cyclodextrin (79).

Our experiments demonstrated that membrane cholesterol was required for JEV infection of both neuronal cells and fibroblasts. Cholesterol depletion was done by treating cells with methyl-β-cyclodextrin, a drug that selectively extracts cholesterol from the plasma membrane (37), or with filipin, a compound that binds selectively to cholesterol, forming complexes in the plasma membrane that sequester cholesterol and induce structural disorder (10). Cells were treated with 5mM and 7.5mM methyl-β-cyclodextrin, or with 0.5µM and 1µM filipin before infecting them with JEV. Both treatments resulted in a marked inhibition (60% or greater) of JEV infection highlighting an essential role for cholesterol in both clathrin dependent and clathrin independent endocytosis of JEV in different cell lines (Figure
Quantitative RT-PCR also showed an inhibition of JEV endocytosis in cholesterol depleted Neuro2a cells (Figure 6B).

**JEV binding on cells leads to active actin rearrangements**

The actin cytoskeleton is an important regulator of all membrane processes. Studies have shown that upon interaction with filopodia, several viruses like HIV, MLV and VSVG undergo rapid actin- and myosin II- driven transport, “surfing” to entry sites at the cell body (45). To test whether JEV binding and infection events lead to any global actin rearrangements, virus was added to Neuro2a cells for different times and actin was observed via phalloidin staining. Uninfected cell surfaces were smooth and showed a clear margin with peripheral F-actin staining and random filopodial projections (Figure 7A, upper left panel). As early as 3 min post-infection several cells showed increased intensity of cortical actin structures and filopodial projections (Figure 7A, upper right panel), which increased with time (Figure 7A, lower panels). By 5 min post-infection several cells showed a dramatic increase in the number of filopodia that showed a regular arrangement around the cell periphery (Figure 7A, lower left panel). This effect peaked at 10 min post-infection (Figure 7A, lower right panel). This increased distribution of filopodia on cells is likely to represent induction of an efficient infectious pathway brought about by some signalling event initiated by virus binding.

**JEV entry in neuronal cells is actin and myosin II dependent**

The induction of actin-rich filopodia on cells after virus binding, imply that viral-entry is likely to be an actin and motor-driven process. We further examined the role of the actin cytoskeleton in JEV entry by using chemical inhibitors of actin polymerization and depolymerization. Cytochalasin D (CytoD) reversibly targets barbed ends of F-actin, inducing depolymerisation of existing filaments and increasing the cellular pool of ADP-
bound actin monomers (30). Latrunculin A reversibly disrupts actin dynamics by targeting monomeric G-actin and preventing actin polymerization (75). Treatment of cells with CytoD at a concentration of 5µM and 2.5µM, showed a 70% block in JEV infection in Neuro2a cells, and a 20% decrease in Vero cells (Figure 7B). Similarly, treatment with Latrunculin at concentrations of 5µM and 2.5µM significantly reduced JEV infection in Neuro2a cells while having a marginal effect in Vero cells (Figure 7B). To block turnover of actin microfilament we used a cell permeable drug jasplankinolide, that selectively binds to F-actin, and dramatically decreases rate of actin depolymerization (12). Treatment with jasplankinolide at concentrations ranging from 0.5 to 2µM resulted in about 80% inhibition of JEV infection in Neuro2a cells (Figure 7B). We also confirmed that these drugs block JEV entry inside cells by qPCR (Figure 7C). Collectively, our pharmacological and immunofluorescence labelling data strongly suggests that the dynamic reorganization (depolymerization/ repolymerization) of actin microfilaments is critical for JEV entry in neuronal cells.

We further examined whether myosin driven contractions would provide the mechanical force for JEV infection process. It has been shown that myosin motors are involved in movement of actin filaments in cell surface protrusions (67), a process known as rearward or retrograde F-actin flow. Nonmuscle myosin II is a plus-end motor that localizes to the lamellum and retraction fibres, and is involved in viral surfing on filopodia. Since myosin II is an important contributor to the cytoskeleton of neuronal cells, and may influence the trafficking of JEV, we tested its involvement in viral entry by using its specific inhibitor blebbistatin (76). Blebbistatin interferes neither with binding of myosin to actin nor with ATP-induced actomyosin dissociation, but blocks myosin II in an actin detached state. Treatment of cells with blebbistatin (50µM) resulted in about 50% block in JEV infection in Neuro2a cells and a 30% block in Vero cells (Figure 6B, C). These results suggest that...
myosin is essential for infection and JEV entry may involve virus movement along filopodia to the cell body utilizing the underlying actin-myosin II machinery.

**Role of the small GTPases in JEV infection**

We next examined the role of the Rho family of small GTPases- Rho, Rac and Cdc42 that are involved in spatio-temporal control of actin polymerization and also play a regulatory role in endocytic processes (27). To dissect the effects of individual Rho family GTPases we expressed wild-type, dominant-negative and dominant-active isoforms of RhoA, Rac1 and Cdc42 and tested their activity in cells (Figure 8A, B, C). While the dominant-active isoforms showed several fold higher GTPase activity over wild-type, the dominant-negative isoforms showed close to 50% decrease in GTPase activity when over-expressed. These constructs were expressed in Neuro2a cells and checked for their effect in the JEV infection process. Cells expressing myc-tagged isoforms of RhoAwt, Cdc42wt, Cdc42N17 (DN), Cdc42L61(DA), Rac1L61(DA) and Rac1N17(DN) had roughly equal efficiency of JEV infection. Only the RhoA-N19 (DN) isoform showed about a 50% reduction in JEV infection (Figure 8D). The dominant active RhoA (L63) had a minor effect and reduced JEV infection by about 20%. This could be because of decreased GTP-GDP turnover rates of endogenous Rhowt protein in the background of over-expression of dominant active DA. As an alternative we also tested the effect of CT04, a specific RhoA inhibitor (8, 13). The active site of CT04 is the exoenzyme C3 Transferase from *Clostridium botulinum*. CT04 specifically inhibits RhoA, B and C proteins by ADP-ribosylation on asparagine 41 in the effector binding domain of the GTPase (4). Rho inhibition by CT04 was tested in Neuro2a cells by cell morphology phenotypes and phalloidin staining. Cells treated with CT04 at a concentration of 2µg/ml showed close to 60% block in JEV infection and a 50% block in JEV entry, strongly suggesting the involvement of RhoA in the JEV endocytic internalization process in neuronal cells (Figure 8F, G).
Since RhoA appeared to play a critical role in viral infection, we investigated the activation status of RhoA during the initial stages of JEV entry in Neuro2a cells. JEV (10MOI) was used to infect cells, and Rho activation was measured with the G-LISA Rho activation kit following the manufacturer’s instructions. The results showed that Rho activation is an immediate and robust event during JEV entry, reaching a maximum (close to 2 fold) at 10mpi compared to mock infected cells (Figure 8E). This result further corroborates that JEV entry induces RhoA activation in neuronal cells.

JEV infection requires passage through Rab5 positive early endosomes, but is independent of Rab7

It is established that all flaviviruses require acidification in the endosome for uncoating and release of the viral nucleic acid for replication (55, 60). We confirmed that JEV entry in Neuro2a cells was acid dependent, as pre-treatment of cells with 100-200nM Bafilomycin A1—a drug that is a potent inhibitor of the vacuolar ATPase and specifically prevents acidification of endosomal vesicles (11), led to about 95% decrease in JEV infection in both Neuro2a and Vero cells (Figure 9B).

Rab proteins are known to orchestrate membrane traffic in the cell, and show distinct localizations to specific subcellular compartments. Cargo from CME is sorted into early endosomes that are marked by Rab5 and EEA1. From the early endosomes molecules are either sorted for recycling or directed to late endosomes that are Rab7 and LAMP1 positive. Endosomes carrying cargo internalized via clathrin independent pathways also fuse with Rab5 positive early endosomes (35, 59). Studies with Dengue virus have demonstrated that the virus moves from Rab5 positive early endosomes to Rab7 positive late endosomes before viral RNA is released in the cytoplasm (55, 80). West Nile virus was also shown to traffic from EEA1 positive early endosomes to LAMP1 positive late endosomes where virus
uncoating finally occurred (18, 19). In contrast, another study based on RNA interference showed entry of Dengue and West Nile virus to be dependent on Rab5 but not on Rab7, indicating that virus uncoating occurs at the level of early endosomes (40).

To investigate the endocytic trail followed by JEV, DiD labelled virus was allowed to bind to Neuro2a cells transfected with either dsRed-Rab5 or GFP-Rab7, on ice for 1 hour, and then incubated at 37°C for different time points to follow virus internalization in the cell. Virus internalization was a slow process and virus particles were seen in endosomes distinct from Rab5 till 20 min of internalization (Figure 8A, upper panels). After 30 min of uptake virus containing vesicles had acquired/ fused with Rab5 endosomes (Figure 8A, lower left panel, arrowheads). By 1h of internalization virus signal significantly decreased and was no longer detectable inside the cell. Labelled virus particles were not detected in GFP-Rab7 positive late endosomes at any time point, indicating virus fusion is happening at a level upstream of Rab7 late endosomes (Figure 8A, lower right panel).

To further confirm this observation, Neuro2a and Vero cells were transfected with GFP-dnRab5 or GFP-dnRab7 for 24 h and virus infection was monitored. Whereas cells transfected with dnRab5 showed about 90% block in JEV infection, cells transfected with dnRab7 showed no significant inhibition. This indicates that JEV fusion occurs at the level of the early endosome and not the late endosome (Figure 9C).

**Discussion**

Current knowledge indicates that a complex network of diverse, continuing and triggered pathways operate at the eukaryotic plasma membrane. Cargo specificity, coat proteins and scission molecules are used to define most of these mechanisms. The orderly transport of endocytic cargo is tightly regulated and requires the participation of numerous lipids and accessory proteins. Endocytosis also requires alterations of fine cellular structures.
and mechanical force to internalize a vesicle. Viruses are adept at exploiting these mechanisms for gaining entry (50). Nearly all viruses utilize an endocytic mechanism to gain entry into a permissive cell and establish infection. These pathways serve to deliver viruses to vesicles and compartments conducive to viral membrane fusion and release of the core into the cell cytoplasm at a site permissive to replication. The route of virus entry can differ between cell types. In addition to utilizing the already operational endocytic pathways, in several cases viruses can induce pathways conducive to entry by receptor binding and signalling events.

The results presented here indicate that entry of JEV in neuronal cells involves strategies different from those described for many other flaviviruses. JEV internalization occurs through a clathrin independent mechanism, and the cellular factors needed for entry are dynamin, cholesterol, dynamic actin cytoskeleton, the small GTPase RhoA and myosin II motors. After endocytosis, the virus trafficks through Rab5 positive early endosomes which are possibly the sites for viral uncoating and genome release.

The information currently available suggests that flavivirus internalization is clathrin dependent. Studies have shown that JEV infection of Vero and neural stem/progenitor cells can be inhibited by chlorpromazine (24, 62). We also observed a clathrin dependent pathway for JEV infection in Vero cells. By depleting the key coat proteins- clathrin heavy chain and clathrin light chain, our study clearly demonstrates that a clathrin independent pathway is operational for JEV entry in neuronal cells. Neuronal cells are highly relevant for investigating JEV infection, since JEV is primarily a neurotropic virus. It is likely that the endocytic route followed by the virus is cell type dependent.

Cholesterol dependence is often considered to be a marker of caveolar/ lipid-raft mediated pathway (29). However, cholesterol is essential for most membrane processes. For
flaviviruses as well, membrane cholesterol is a requirement for entry (24, 53). Our study with different cell types shows that membrane cholesterol is an absolute requirement for JEV internalization.

JEV was seen associated with filopodia and the cell body. Virus binding induced dynamic rearrangements of the actin cytoskeleton at the early stages of infection. Several viruses activate small GTPases to form lamellipodia and filopodia in cells (20, 28). Our data from experiments with drugs that alter actin organization show that both actin polymerization and depolymerization are required during the virus infection cycle. Filopodia are connected to the cortical actin network which, in association with myosin II, controls their contractility at their base, and the motor activity of myosin II could be required for JEV infection. Our results point out to a greater role for the actin cytoskeleton in early viral entry events in neuronal cells.

After receptor binding, viruses undergo a period of diffusive or directed motion on the plasma membrane, until they become confined (14). Some viruses can move along filopodia to internalization sites by receptor interaction with the actin cytoskeleton and retrograde flow within filopodia (45, 73). The actin cytoskeleton plays a structural role in endocytosis. Its dynamic nature is essential for its function. Existing actin filaments undergo severing and depolymerisation in response to cellular requirements and stimuli, while new actin filaments are polymerized from monomeric actin subunits and by branching off from existing filaments. These processes are regulated by the Rho GTPases. Studies on Herpes viruses have shown that RhoA can induce filopodia in infected cells (20). Kaposi’s sarcoma-associated herpesvirus (KSHV) entry induces RhoA GTPase, and rearrangements of both microtubules and the actin cytoskeleton in fibroblasts (74), and RhoA GTPase is also important for virus entry in HEK293 cells (81). Our study also points to an important role for RhoA in virus entry.
entry. The binding of JEV to its specific receptor is likely to activate signalling of cells via RhoA activation.

Several viruses induce macropinocytosis and use it as a route for internalization and infection (25, 36, 54, 57). JEV binding and uptake in Neuro2a cells did not show characteristics of macropinocytic uptake. Further JEV internalization was not affected by treatment of cells with amiloride, an inhibitor that specifically blocks Na+/H+ exchange and macropinocytosis (data not shown).

The present findings provide the first evidence for a clathrin-independent, actin-dependent pathway utilized by JEV for infection of neuronal cells. At least three clathrin-independent mechanisms have been described at the plasma membrane, primarily on the basis of cargo, dynamin dependence/independence, role of cellular factors like Arf and Rho GTPases, actin and cholesterol requirements (39, 52). It is still unclear whether JEV utilizes a pre-existing clathrin independent endocytic pathway or a specific pathway is induced in response to virus binding and downstream signalling. The receptor for JEV on neuronal cells is still uncharacterized. It is possible that binding to a specific receptor and entry through a clathrin independent pathway confers the virus with a greater advantage to establish infection in neuronal cells. Like all viruses, JEV relies on host cell factors and physiological processes for key steps of its replication cycle. Identification of these processes and factors will not only allow a better insight into pathogenic mechanism, but may identify novel targets for future therapeutic development.
Acknowledgements

This work was supported by Department of Biotechnology (DBT), Government of India grant- BT/MB/01/VIDRC/08 to SV.

We would like to thank Dr. Alexandre Benmerah for the Eps15 constructs. MK is grateful to Drs. Shahid Jameel, Sankar Bhattacharya and Arup Banerjee for critical inputs on the manuscript. We thank Drs. Manpreet Kaur, Vikas Sood and Taranjeet Kaur for assistance with flow cytometry, RT-PCR and virus generation.
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Figure 1. JEV internalization is dynamin dependent. (A) Neuro2a cells grown on coverslips were either untreated, or treated with 80 µM dynasore for 1h, following which they were infected with JEV (MOI 0.4) in the presence of the inhibitor. 24 hpi cells were fixed and stained with anti-JEV E primary and Alexa488 anti-mouse IgG secondary antibodies. Cells were mounted in DAPI containing anti-fade and imaged at 20X on a confocal microscope. Cell nuclei can be seen in blue (DAPI) and JEV-E in green. Bar, 20µm. (B) Neuro2a cells were infected with JEV (MOI 10) in the presence of 80 µM dynasore for 1h, following which the endocytosed viral load was estimated by qRT-PCR of JEV positive strand RNA. (C) Quantitation of JEV infection (MOI 0.4) in Neuro2a, SH-SY5Y and Vero cell lines in the presence of 80 µM dynasore was done by microscopy and image analysis as described in Materials and Methods. Depicted results are normalized to solvent treated control cells. (D) After 24hpi (MOI 0.4) the virus titre in culture supernatants was calculated by plaque assay’s (mean ± S.D.) (E) Neuro2a cells transfected with GFP, or GFP-dyn2-K44A were infected with JEV (MOI 1, ~ 30% infection in GFP-transfected cells). Cells were fixed at 24 hpi, stained with anti-JEV E primary and Alexa568 anti-mouse IgG secondary antibody, and imaged at 60X on a confocal microscope. Bar, 10µm. (F) JEV infection in Neuro2a, SH-SY5Y and Vero cells expressing GFP and GFPdyn2K44A was quantitated as described above. Infection was normalized to GFP transfected cells. (B, C and F) Student’s t test was used to generate p values; **p<0.01, *p<0.05.

Figure 2. DiD-JEV is internalized independently of clathrin mediated endocytosis cargo-transferrin. (A) DiD-JEV particles were spotted onto polyD-lysine coated coverslip, immunostained with JEV E antibody and imaged on a 60X objective. Images are presented as gray-scales for individual colours, and pseudocoloured as indicated prior to being merged.
Inset shows a magnified view of the region corresponding to the asterix (*) Bar, 10µm. (B) DiD JEV and unlabelled JEV were processed identically and infectious viral titres were calculated by plaque assays. (C) DiD JEV (pseudocoloured in green) was allowed to bind to Neuro2a cells on ice for 1h, washed with chilled PBS, and then incubated at 37°C for different times in the presence of Alexa568/Alexa647-Tf (pseudocoloured in red). Cells were imaged at 60X. Upper right panel represents a DIC image (superimposed with fluorescence from DiD channel, pseudocoloured in green), of the fluorescent image in upper left panel. Note that DiD JEV endosomes remain segregated from Tf endosomes till 15 min (arrowheads) and some overlap is detected only by 30 min (indicated by arrows). Bar, 5µm.

Figure 3. JEV entry and infection in neuronal cells is not inhibited by chlorpromazine treatment. (A) Neuro2a, SH-SY5Y and Vero cells were pre-treated for 30 min with chlorpromazine at the indicated concentrations, and given a pulse of Alexa488 Tf for 10 min. Cells were fixed and transferrin uptake was quantified by flow cytometry. The average of measured geometric means of internalized Tf in control and chlorpromazine treated cells is shown ± S.D. (B) Neuro2a, SH-SY5Y and Vero cells were pre-treated for 30 min with 50µM chlorpromazine and infected with JEV (MOI 0.4) in the presence of inhibitor. Infection was scored as described in Materials and Methods. Student’s t test was used to generate p values; *p<0.05. (C) Neuro2a cells were infected with JEV (MOI 10) in the presence of 50 µM chlorpromazine for 1h and the amount of virus endocytosed was estimated by qRT-PCR of JEV positive strand RNA. (D) Virus titres in culture supernatants 24hpi (MOI 0.4) were calculated by plaque assay’s (mean ± S.D.)

Figure 4. JEV infection in neuronal cells is clathrin independent. (A, B) Neuro2a cells were transfected with plasmids- GFP Eps15-DIIIδ (A) and GFP Eps15-DIII (B), and 24 h later given a pulse of Alexa568 Tf for 10 min. Images are presented as gray-scales for individual colours, and pseudocoloured as indicated prior to being merged. Note that majority
of cells transfected with Eps15-DIII (arrows) show a block in Tf uptake whereas cells
transfected with control Eps15- DIIIδ2, (arrows) show Tf uptake. Bar 20µm. (C) Quantitation
of Tf internalization in Neuro2a cells transfected with GFP-Eps15 constructs- DIIIδ2 and
DIII was done as described in Materials and Methods. (D) Neuro2a, SH-SY5Y and Vero cells
transfected with GFP-Eps15-DIIIδ2 and GFP-Eps15 DIII were infected with JEV (MOI 1).
24 hpi, cells were fixed and stained for JEV E antigen. Infection was scored by counting
number of GFP transfected cells stained vs unstained for JEV E and normalized to GFP
expressing infected cells. (E) Virus titres in culture supernatants of transfected cells 24hpi
(MOI 1) were calculated by plaque assay’s (mean ± S.D.). (C and D) Student’s t test was
used to generate p values; **p<0.01, *p<0.05.

Figure 5. Depletion of clathrin light chain and clathrin heavy chain proteins has no
effect on JEV infection of neuronal cells. (A) Neuro2a cells were transfected with either
GFP-Retro Q shRNA plasmid vector alone (left panel) or GFP-CLC shRNA (right panel) and
72 h later given a 10 min pulse of Aexa647 Tf (pseudocoloured in red). Note that cells
expressing vector alone show efficient Tf uptake while cells expressing CLC shRNA show a
block in Tf endocytosis. Bar 5 µm. (B) Quantitation of Tf uptake was done as described in
Materials and Methods. (C) Western blots showing depletion of CLC in Neuro2a and Vero
cells transfected with CLCshRNA plasmid for 72h, and loading control-actin. (D) Neuro2a
and Vero cells were transfected with shRNA plasmids directed against clathrin light chain
(CLC) or control mock plasmid. After 72 h cells were infected with JEV (MOI 1), and scored
24 hpi by immunofluorescence staining of JEV E antigen. Infection was quantified as
described above. (E) JEV titres in Neuro2a and Vero cells transfected with control and
CLCshRNA plasmid, estimated 24hpi (MOI 1). (F) Western blot showing depletion of CHC
in in Neuro2a cells transfected with shRNA plasmids for 72 h, and loading control-actin. (G)
JEV infection in Neuro2a cells in the background of CHC depletion was scored as described above. (H) Virus titres in culture supernatants of Neuro2a cells depleted of CHC, infected at MOI 1 was calculated 24hpi by plaque assays (mean ± S.D.). Student’s t test was used to generate p values; **p<0.01, *p<0.05.

**Figure 6. Cholesterol is required for JEV infection.** (A) Neuro2a and Vero cells were treated with indicated concentrations of MβCD or Filipin for 1 h and infected with JEV (MOI 0.4) in the presence of inhibitor. 24 hpi cells were fixed, and infection was quantified as described in Materials and Methods. (B) Neuro2a cells were treated with indicated concentrations of MβCD or Filipin for 1 h and infected with JEV (MOI 10) in the presence of inhibitor. Endocytosed viral load was estimated by qRT-PCR of JEV positive strand RNA. Student’s t test was used to generate p values; **p<0.01, *p<0.05.

**Figure 7. Role of actin and myosin motors in JEV entry in neuronal cells.** (A) JEV (10MOI) was added to Neuro2a cells for indicated time points at 37°C, following which cells were washed with chilled PBS, fixed and stained with Alexa546 Phalloidin. Images were taken at 60X. Bar, 10µm. (B) Neuro2a and Vero cells were treated with inhibitors-cytochlasin D, latrunculin, jasplakinolide and blebbistatin at the indicated concentrations. Cells were infected with JEV (MOI 0.4) in the presence of inhibitor, and infection was scored as described in Materials and Methods. (C) Neuro2a cells with treated with inhibitors at the indicated concentrations and infected with JEV at 10MOI in the presence of the inhibitor. Virus entry was quantified by qRT-PCR. Student’s t test was used to generate p values; **p<0.01, *p<0.05.

**Figure 8. The GTPase Rho A is required for JEV infection of neuronal cells.** (A- C) Neuro2a cells were transfected with myc-tagged constructs of RhoA wt, RhoA L63, RhoA N19, Rac1 L61, Rac1 N17, Cdc42wt, Cdc42 L61 and Cdc42 N17. The GTPase activity of the
over-expressed constructs was quantified using GTPase specific ELISA as described in Materials and Methods. (D) Neuro2a cells transfected with the wt, DA and DN constructs of Rho, Rac and Cdc42 were infected with JEV (MOI1). 24 hpi double immuno-fluorescence staining was done for Myc and JEV E. Cells staining positive for both Myc- and JEV E were scored and normalized to RhoA wt expressing infected cells. (E) Time course of Rho activation in response to JEV binding was quantified using RhoGTPase specific ELISA as described in Materials and Methods. (F) Neuro2a cells were serum starved for 2h before adding CT04 inhibitor to cells for another 2h. Cells were infected with JEV (MOI 0.4) in the presence of inhibitor. Infection was quantified as described in Materials and Methods. (G) Virus endocytosis (10MOI) was quantified in control and CT04 treated cells by qRT-PCR.

Student’s t test was used to generate p values; **p<0.01, *p<0.05.

Figure 9. JEV infection requires trafficking through Rab5 positive compartments but is independent of Rab7. (A) DiD JEV (pseudocoloured in green) was added to Neuro2a cells transfected with dsRed-Rab5 or GFP-Rab7 (pseudocoloured in red), on ice for 1 h. Cells were warmed to 37°C for indicated times, washed with low pH buffer, fixed and imaged. DiD JEV colocalization with Rab5 is seen only by 30min post internalization (lower left panel, arrowheads). Bar, 10µm. (B) Neuro2a and Vero cells were pre-treated with 100nM Bafilomycin before infection with JEV. Infection was quantified as described in Materials and Methods. (C) Neuro2a and Vero cells transfected with GFP, GFPRab5wt, GFPRab5dn, GFPRab7wt, GFPRab7dn were infected with JEV and processed 24 hpi as described above. Infection was normalized to cells transfected with GFP alone. Student’s t test was used to generate p values; **p<0.01.
**A**

Normalized Geometric Mean of fluorescence intensity (a.u.)

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<td>Vero</td>
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**B**

JEV infected cells (% of control)

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<tr>
<td>Vero</td>
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**C**

JEV internalization (relative to control)

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<thead>
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<th></th>
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</thead>
<tbody>
<tr>
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**D**

**Virus Titre**

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<th>Vero</th>
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<td>Chlorpro.</td>
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<td>3.1 X 10^5 ± 0.8X10^6</td>
<td>6.6 X 10^4 ± 0.8X10^4</td>
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Figure A: JEV infected cells (% of control) for different concentrations of MβCD and FIL.

Figure B: JEV internalization (relative to control) for different concentrations of MβCD and FIL.
A

DAPI/Phalloidin

0'

3'

5'

10'

B

JEU infected cells (% of control)

Neuro2a

Vero

Control 2.5 μM CytoD 5 μM CytoD 2.5 μM LatA 5 μM LatA 1 μM Jas 2 μM Jas 17 μM Blebbi 25 μM Blebbi

C

JEU internalization (relative to control)

Control 5 μM CytoD 5 μM LatA 2 μM Jas 25 μM Blebbi