Journal of Neurochemistry

JOURNAL OF NEUROCHEMISTRY | 2010 | 115 | 537–549 doi: 10.1111/j.1471-4159.2010.06951.x

Critical role of lipid rafts in virus entry and activation of phosphoinositide 3′ kinase/Akt signaling during early stages of Japanese encephalitis virus infection in neural stem/progenitor cells

Sulagna Das, Swarupa Chakraborty and Anirban Basu

National Brain Research Centre, Manesar, Haryana, India

Abstract

Japanese encephalitis virus (JEV), the leading cause of acute encephalitis in South-East Asia is a neurotropic virus infecting various CNS cell types. Most Flaviviruses including JEV get internalised into cells by receptor-mediated endocytosis, which involve clathrin and membrane cholesterol. The cholesterol-enriched membrane microdomains referred to as lipid rafts act as portals for virus entry in a number of enveloped viruses, including Flavivirus. However, the precise role played by membrane lipid rafts in JEV internalisation into neural stem cells is still unknown. We have established neural stem/progenitor cells and C17.2 cell line as models of productive JEV infection. Increase in membrane fluidity and clustering of viral envelope proteins in lipid rafts was observed in early time points of infection. Localisation of non-structural proteins to rafts at later infection stages was also observed. Co-localisation of JEV glycoprotein with Cholera toxin B confirmed that JEV internalisation occurs in a lipid-raft dependent manner. Though JEV entry is raft dependent, however, there is requirement of functional clathrin during endocytosis inside the cells. Besides virus entry, the lipid rafts act as signalling platforms for Src tyrosine kinases and result in activation of phosphoinositide 3′-kinase/Akt signalling during early JEV infection. Disruption of lipid raft formation by cholesterol depletion using Methyl β-cyclodextrin, reduced JEV RNA levels and production of infectious virus particles as well as impaired phosphoinositide 3′-kinase/Akt signalling during initial infection. Overall, our results implicate the importance of host membrane lipid rafts in JEV entry and life cycle, besides maintaining survival of neural stem/progenitor cells during early infection.

Keywords: cholesterol, Japanese encephalitis virus, lipid rafts, neural stem/progenitor cells.

J. Neurochem. (2010) **115**, 537–549.

Japanese encephalitis virus (JEV) belonging to the family *Flaviviridae* are important human pathogenic viruses causing a variety of diseases ranging from mild febrile illness, to hemorrhagic fever to acute encephalitis (Mackenzie *et al.* 2002; Ghosh and Basu 2009). The virus infects all age groups with higher incidence in children, causing fatal encephalitis in 25–40% of cases and long term neurological sequelae in 50% of those who survive (Diagana *et al.* 2007; Myint *et al.* 2007). Flaviviruses like JEV and West Nile Virus (WNV), are neurotropic, and infect various cell types in the CNS. Flavivirus adsorption on cell surface induces changes in the host membrane to promote virus entry (Anderson 2003). JEV entry occurs via attachment of the virions on the cell surface, followed by internalization by receptor-mediated endocytosis (Nawa *et al.* 2003). The host membrane plays critical role in various stages of the viral life cycle- from entry, replication, assembly and egress of the virus particles.

Animal membranes may be segregated into a liquid ordered phase (L_o) enriched in cholesterol and sphingomyelin and a disordered phase. The L_o phase form small, dynamic and transient structures in the membrane referred to as the ‘rafts,’ which can be isolated as detergent resistant

Received June 23, 2010; revised manuscript received August 4, 2010; accepted August 6, 2010.

Address correspondence and reprint requests to Anirban Basu, National Brain Research Centre, Manesar, Haryana-122050, India. E-mail: anirban@nbrc.ac.in

Abbreviations used: CTB, cholera toxin B; DENV, Dengue virus; DMEM, Dulbecco’s modified Eagle’s media; DPH, 1,6-diphenyl-1,3,5 hexatriene; FAK, Focal adhesion kinase-1; GFP, green fluorescent protein; JEV, Japanese encephalitis virus; MBCD, methyl β-cyclodextrin; MEM, minimum essential media; NSPC, neural stem/progenitor cell; PBS, phosphate-buffered saline; PFA, paraformaldehyde; PI3K, phosphoinositide 3′-kinase; SFK, Src-family kinase; WNV, West Nile virus.

membranes (DRMs) from cells (Brown 2006). The resistance of lipid rafts to non-ionic detergents like Triton X-100 at low temperature is attributed to their tight packing or organisation in the membranes, and is critically dependent on the presence of cholesterol (Brown and London 1998). A number of enveloped viruses like Retroviruses, Filoviruses, Alphaviruses and Flaviruses have been shown to utilise rafts for productive infection at various stages of their life cycle- from virus entry, assembly and egress (Chazal and Gerlier 2003; Suzuki and Suzuki 2006). The rafts facilitate virus entry either by: (i) concentrating the receptors required for binding and/or oligomerization of the virus envelope proteins or (ii) by inducing signal transduction upon binding of the viral protein (Stiasny *et al.* 2003).

In Flaviviral infections, the involvement of membrane cholesterol and lipid rafts has been investigated in WNV and Dengue virus (DENV) infections especially during entry and replication stages. The requirement of cholesterol in virus entry and life cycle is still controversial as supplementation of cholesterol at early stages of infection blocked the adsorption of virions and infectivity of DENV serotype 2 and JEV (Lee *et al.* 2008). In WNV, however, redistribution of membrane cholesterol occurs and changes in cholesterol biosynthesis/trafficking affected WNV RNA replication (Mackenzie *et al.* 2007). Though some reports suggest the role of lipid rafts and membrane cholesterol in JEV life cycle (Lee *et al.* 2008), however, the association of virus proteins with lipid rafts during entry and replication and the role of rafts in early infection have not been investigated.

One of the other important properties of lipid rafts is their ability to include or exclude proteins to variable extents and thereby act as signalling platforms for a number of cellular processes (Simons and Toomre 2000; Golub *et al.* 2004). One such class of proteins with high affinity towards rafts is that of Src-family kinases (SFKs). These non-receptor tyrosine kinases have been involved in cellular proliferation through the Ras-MAPK pathway and in cell survival through the phosphoinositide 3′-kinase (PI3K)/Akt pathway (Brown and Cooper 1996; Yeatman 2004). The association of Src with lipid rafts has been shown to be correlated with their ability to signal to PI3K and lead to subsequent activation of protein kinase B (PKB)/Akt signaling (de Diesbach *et al.* 2008). A number of viruses activate PI3K/Akt signaling in a bid to slow down apoptosis of the infected cell and prolong viral replication in both acute and persistent infections (Cooray 2004). Such activation of the PI3K/Akt pathway and inhibition of cell death pathway has been observed in Flaviviral infections like JEV (Lee *et al.* 2005).

We have previously reported that JEV infects neural stem/progenitor cells (NSPCs) and impair their proliferation (Das and Basu 2008). As NSPCs show permissiveness to JEV, it is important to elucidate the mechanism of virus entry into these stem cells. The exact role of membrane fluidity and lipid rafts in JEV entry into NSPC has not been explored. Moreover, as lipid rafts act as platforms mediating signal transduction, therefore, we wanted to investigate how these rafts modulate SFK signalling during early JEV infection of NSPCs.

Materials and methods

Cell culture

Mouse neural stem cell line C17.2 was a kind gift from Dr. Jaewon Lee, Pusan National University, Korea (originally obtained from Prof. Constance L. Cepko, Harvard Medical School, Boston, MA, USA) (Snyder *et al.* 1992). The cells were grown in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% fetal bovine serum and 5% horse serum and antibiotics.

Neurosphere cultures were established from subventricular zone of postnatal day 7 BALB/c mouse pups as described earlier (Das *et al.* 2009). Briefly, subventricular zone dissected out aspectically was enzymatically digested using Papain (2 mg/mL) and DNaseI and then washed twice with serum containing media. Following final washes with serum-free DMEM, cells were suspended in DMEM-F12, supplemented with B27 supplements, 50 μg/mL gentamycin (all from Gibco, Carlsbad, CA, USA) and growth factors Epidermal Growth Factor (20 mg/mL) and basic Fibroblast Growth Factor (10 mg/mL) (R&D Systems, Minneapolis, MN, USA). Fresh media was added after every 3 days and fully-grown spheres were dissociated using Accutase (Sigma, St. Louis, MO, USA) and all treatment was done after two passages. All animal experiments were done in accordance to protocols approved by Institutional Animal and Ethics Committee of National Brain Research Center (NBRC/IAEC/2008/41).

Virus infection and cell treatment

GP78 strain of JEV was isolated from brain homogenates of BALB/c mouse pups and virus titres were determined using monolayers of PS (porcine kidney) cells as described earlier (Vrati *et al.* 1999; Swarup *et al.* 2007).

C17.2 cells grown to confluence, and neurospheres (density of 10^5 cells/cm^2) after 24 h of plating were infected with JEV at multiplicity of infection of 5 for 1 h at 37°C. Following infection, the cells were washed twice with phosphate-buffered saline (PBS) and then fresh media was added. The supernatant from the infected cells were collected at different hour post-infection (h p.i.) and plaque assay was done using this supernatant. Monolayer of PS cells was incubated with different dilutions of the culture supernatant for 1 h at 37°C. After removal of the supernatant media and PBS washes, monolayers were overlaid with minimum essential media (MEM) containing 4% fetal bovine serum, 1% low melting point agarose and antibiotics. After 72–96 h p.i., once cytopathic effects were seen, the cells were fixed with 4% paraformaldehyde (PFA) and stained with Crystal violet. Plaques were counted and virus titre in terms of plaque forming units (pfu)/mL was determined.

For cholesterol depletion experiments, 5 mM of methyl β-cyclodextrin (MBCD) (Sigma) was added for 1 h prior to JEV infection or 1 h p.i., and then washed twice with media. The dose of MBCD and cell viability upon MBCD treatment was determined previously by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay. Briefly, cells plated in 96-well plates were JEV infected and treated as mentioned above, and 24 h p.i., MTS solution (Promega,

Madison, WI, USA) was added (20 μL in each well). Following incubation for 2–3 h, absorbance was measured at 490 nm and percentage of cell survival was calculated, with control cells considered 100% viable.

Immunocytochemistry

C17.2 cells were grown in Labtek 8-well chamber slides (Nunc, Roskilde, Denmark). Serum free DMEM was added to these cells for 4 h, followed by JEV infection for 1 h as described before. Infection was maintained in the cells both for early time points (1 h) as well as 24 h p.i. In case of neurospheres, both Control and JEV infected NSPCs after 24 h p.i. were dissociated by mechanical trituration and then allowed to adhere on poly-D-lysine-coated 8-well chamber slides for 3 h. Control and infected cells were washed twice with PBS, fixed with 4% PFA for 20 min and blocked in 5% bovine serum albumin for 1.5 h. Cells were then incubated with various primary antibodies – anti-glycoprotein E (gpE; Abcam, Cambridge, UK), anti-αNS1, anti-αNS3 and anti-αNS5 antibodies (a kind gift from Dr. Chun-Jung Chen, Taichung Veterans General Hospital, Taichung, Taiwan) in a humified chamber overnight. A cocktail of anti-gpE (Abcam) and anti-Caveolin1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used for double immunocytochemistry. Respective secondary antibodies, either FITC-conjugated (Vector Labs, Burlingame, CA, USA) or Alexa-Fluor 594 conjugated (Molecular Probes, Eugene, OR, USA) were used and mounted in Vectashield containing 4′-6-diamidino-2-phenylindole (DAPI) (Vector Labs). Images were captured using 20× dry or 40× oil immersion lens in Zeiss Apotome microscope (Carl Zeiss, NY, USA).

Cholera toxin B and transferrin entry assay

C17.2 cells in 8-well chamber slides were incubated with 10 μg/mL of biotin-conjugated cholera toxin B (CTB) (Sigma) in DMEM with or without JEV for 45 min at 4°C. Following attachment of both CTB and virions at 4°C, cells were shifted to 37°C for 30 min to allow endocytosis of both CTB and the virus. Cells were washed with PBS twice, fixed with 2% PFA and then incubated with Streptavidin-conjugated Alexa Fluor 594 (Molecular Probes) for 1 h. Following washes with PBS, cells were then processed for gpE staining as described before.

In another set of experiments, cells were incubated with 15 μg/mL of Alexa 594-labelled Transferrin (Molecular Probes) in DMEM with or without JEV for 45 min at 4°C, and then shifted to 37°C for 45 min. Cells were then fixed in 4% PFA and stained for gpE.

Transfection

C17.2 were seeded in 4-well chamber slides and at 70% confluence, the cells were shifted to antibiotic-free media. Two kinds of clathrin mutants were used: double negative mutant of Eps15 [Green fluorescent protein (GFP)-EΔ95/295], and GFP-D3Δ2 (constructs a kind gift from Prof. A. Benmerah, Institut Pasteur, France) (Benmerah *et al.* 1999). The plasmid DNA (1 μg/mL) and Lipofectamine 2000 (Gibco) were separately incubated in Opti-MEM (Gibco) for 5 min at 24°C, followed by mixing the two solutions and incubating for another 20 min to facilitate formation of liposomal complexes. Total volume of 400 μL of Opti-MEM was added to each chamber, and incubated for 6 h at 37°C. After 6 h, fresh 7% serum containing media was added and left for 24 h post-transfection. Cells were next infected with JEV for 1 h at 37°C, washed thoroughly and then at 6 h p.i. were fixed in 4% PFA and stained for gpE, which was secondary labelled with Alexa 594 (Molecular Probes).

Measurement of membrane fluorescence anisotropy

Control and JEV infected C17.2 cells (at different time points p.i.) were assessed for membrane fluidity using the fluorescent probe 1,6-diphenyl-1,3,5 hexatriene (DPH) as described before (Sharma *et al.* 2007). Briefly, the 2×10^5 cells from each condition was incubated with 1 μmol/L DPH in PBS for 2 h at 37°C with shaking, following which the cells were washed and finally resuspended in PBS. The membrane bound probe DPH was excited at 365 nm and the emission was measured at 430 nm in a Spectrofluorometer (Varian; Netherlands). The fluorescence anisotropy (r) was calculated using the equation $r = [(I\| - I^{\perp})/(I\| + 2I^{\perp})]$, where $I\|$ and $I^{\perp}$ are fluorescence intensities oriented parallel and perpendicular respectively to the direction of polarisation of excited light. The microviscosity parameter $[(r_0/r)-1]^{-1}$ was calculated in each condition, considering the maximal limiting fluorescence anisotropy r_0 to be 0.362 for DPH.

Lipid raft isolation by Opti-prep density gradient centrifugation

Lipid rafts were isolated according to previously described methods (Tewari *et al.* 2008). Control and JEV infected C17.2 cells at different time points were washed in PBS, lysed in 267 μL of ice-cold Optibuffer (50 mM Tris pH 7.5, 150 mM NaCl, 10% sucrose, 1 mM dithiothreitol, 1% Triton-X, 1 mM phenylethyl sulphonyl fluoride, and protease inhibitor mixture). The mixture was kept in ice for 30 min and 533 μL of 60% Optiprep was added to bring the final concentration to 40% Optiprep. The lysates were transferred to ultracentrifuge tubes and sequential layering of 35%, 30%, 25%, 20% and 0% Optiprep diluted in Optibuffer was done on top of the samples to create a gradient. Samples were spun at 30 000 rpm (> 107 000 *g*) in Sorvall S120 ME centrifuge for 18 h at 4°C. After centrifugation, 800 μL-of each fraction was collected from the top in the reverse order of addition. 40 μL of each fraction was mixed with 4 μL of 10× sample buffer, boiled for 5 min and electrophoresed on 10% polyacrylamide gels, and proceeded for western blotting.

Western blotting

Cell lysates were prepared from C17.2 cells and neurospheres at different time points of JEV infection using lysis buffer as described earlier (Ghoshal *et al.* 2007). The concentration of protein was determined using Bradford's reagent and equal amount of protein was loaded in sodium dodecyl sulfate–polyacrylamide gels, transferred onto nitrocellulase membranes and probed with the following antibodies – PI3 kinase p110α, phospho-Akt, Akt (all from Cell Signaling, Danvers, MA, USA), and β-actin (Sigma). Horseradish Peroxidase-conjugated respective secondary antibodies were added. Blots were visualised using chemiluminescence reagent from Millipore (Temecula, CA, USA) in Syngene Gel Documentation System using GeneSnap software. Similarly, blots were developed for the different membrane fractions from control and JEV infected C17.2 cells for the following antibodies: αNS1, αNS3, αNS5 proteins, gpE (Abcam), Nakayama (Millipore), phospho-hemopoietic cell kinase (HCK) (Sigma), p-Src (Santa Cruz), p-phospho-focal adhesion kinase-1 (FAK) (Abcam), Caveolin-1 (Santa Cruz) and CD71 (Zymed Labs, San Francisco, CA, USA).

Semi-quantitative RT-PCR

Control, JEV-infected and treated C17.2 cells were collected in 0.5 mL of Trizol reagent (Sigma) and RNA was prepared according to previously described methods (Ghoshal *et al.* 2007). 500 ng of RNA was used for amplifying GP78 RNA and cyclophilin mRNA with a one-step RT-PCR kit (Qiagen, Hilden, Germany). The PCR primers used have been described before (Swarup *et al.* 2007) and the amplified products were visualised in 2% agarose gels with ethidium bromide.

Results

In vitro infection of C17.2 and mouse neurospheres by JEV

C17.2 and mouse neurospheres either control or JEV infected were cultured for 24 h p.i., fixed and immunostained for JEV specific non-structural (NS) proteins-αNS1, αNS3, αNS5 and the structural glycoprotein E (gpE). Infected C17.2 expressed all the NS proteins and gpE showing that the cell line support active JEV infection (Fig. 1a). Mouse neurospheres at 24 h p.i. dissociated and adhered on poly-D-lysine-coated chamber slides, also showed prominent expression of NS proteins (αNS1, αNS3 and αNS5) along with gpE (Fig. 1b).

It is important to show that JEV replication occurs inside these cells, followed by production of infectious virus particles. We therefore performed plaque assay on PS cells using the culture supernatant from both C17.2 and neurospheres at different h p.i. The virus titre (pfu/mL) in each case was calculated and we observed a progressive increase in plaque formation until 48 h p.i. in C17.2 which reached maximum at 72 h p.i. (Fig. 1c). In infected NSPCs, however, more plaque formation was observed compared to C17.2 cells and increase in the plaque number was observed until

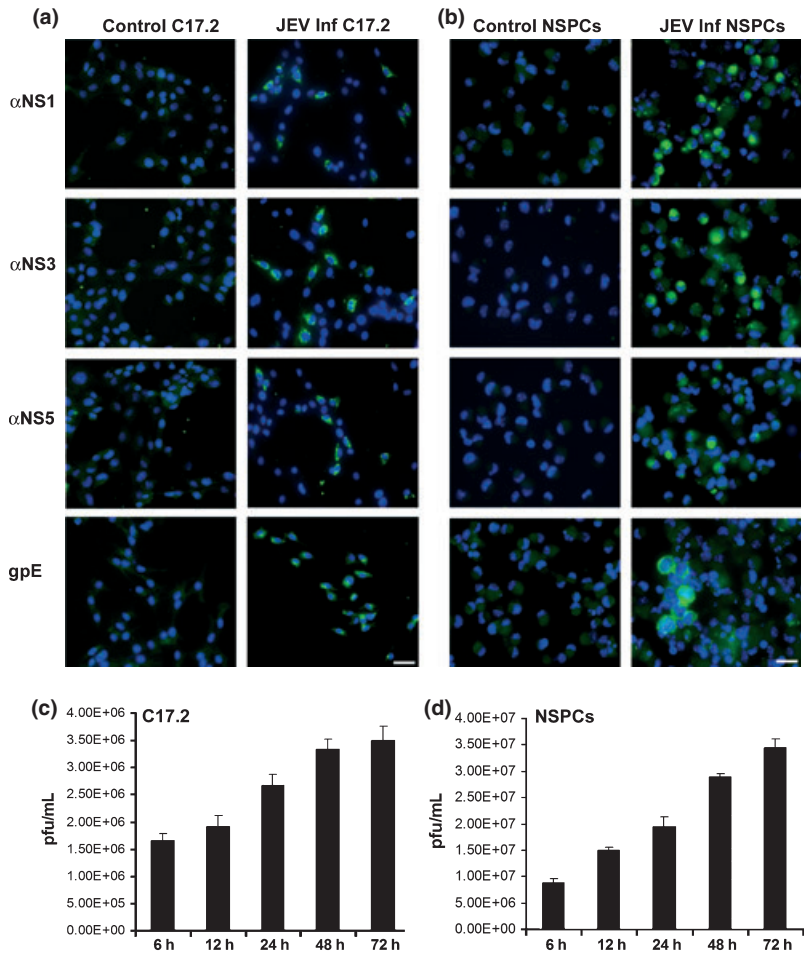

Fig. 1 Productive JEV infection in C17.2 cells and NSPCs. Control and JEV infected C17.2 cells (a) and NSPCs (b) were cultured for 24 h p.i. and stained with JEV non-structural antibodies: anti-αNS1 (top panel), anti-αNS3 (second panel), and anti-αNS5 (third panel) and structural gpE (bottom panel) and acquired under 20× magnification in Zeiss apotome microscope. Expression of JEV NS proteins and gpE was observed in both infected C17.2 cells (a) and NSPCs (b). Scale bar corresponds to 25 microns. Culture supernatant from JEV infected C17.2 (c) and NSPCs (d) at different time points of infection were collected and plaque assay was performed using this supernatant on monolayers of PS cells. Cytopathic effects on PS cells were seen by 72 h and pfu/mL was calculated for each condition. More plaques were formed with JEV infected NSPCs than with C17.2. Values represent mean ± SEM obtained from triplicate.

72 h p.i. (Fig. 1d). Thus, it has been clearly shown that both the primary mouse neurospheres and the stem cell line support JEV replication and produce infectious virus particles.

Membrane fluidity changes in C17.2 cells during early stages of JEV infection

An early event induced by adsorption of virions on host surface is increase in fluidity of cell membranes (Levanon *et al.* 1977) and therefore we investigated whether JEV infection leads to dynamic membrane rearrangements in C17.2 cells. At different time p.i., C17.2 were incubated with the fluorescent probe DPH (1 μmol/L) and the bound probe was excited at 365 nm and emission was recorded at 430 nm. The fluorescence anisotropy value (r) and the microviscosity parameter were calculated as described before (Sharma *et al.* 2007). A significant decrease in membrane microviscosity was observed upon JEV infection as early as 30 min p.i. compared to control cells, and this decrease was maintained until 6 h p.i (Fig. 2a) ($p < 0.01$). As microviscosity is the inverse of fluidity, we can infer that adsorption of JEV virions and its entry into C17.2 leads to an increase in membrane fluidity which is sustained until 6 h p.i., that is, during early infection stages. At later stages of infection, fluidity was restored back to measurements observed in control cells.

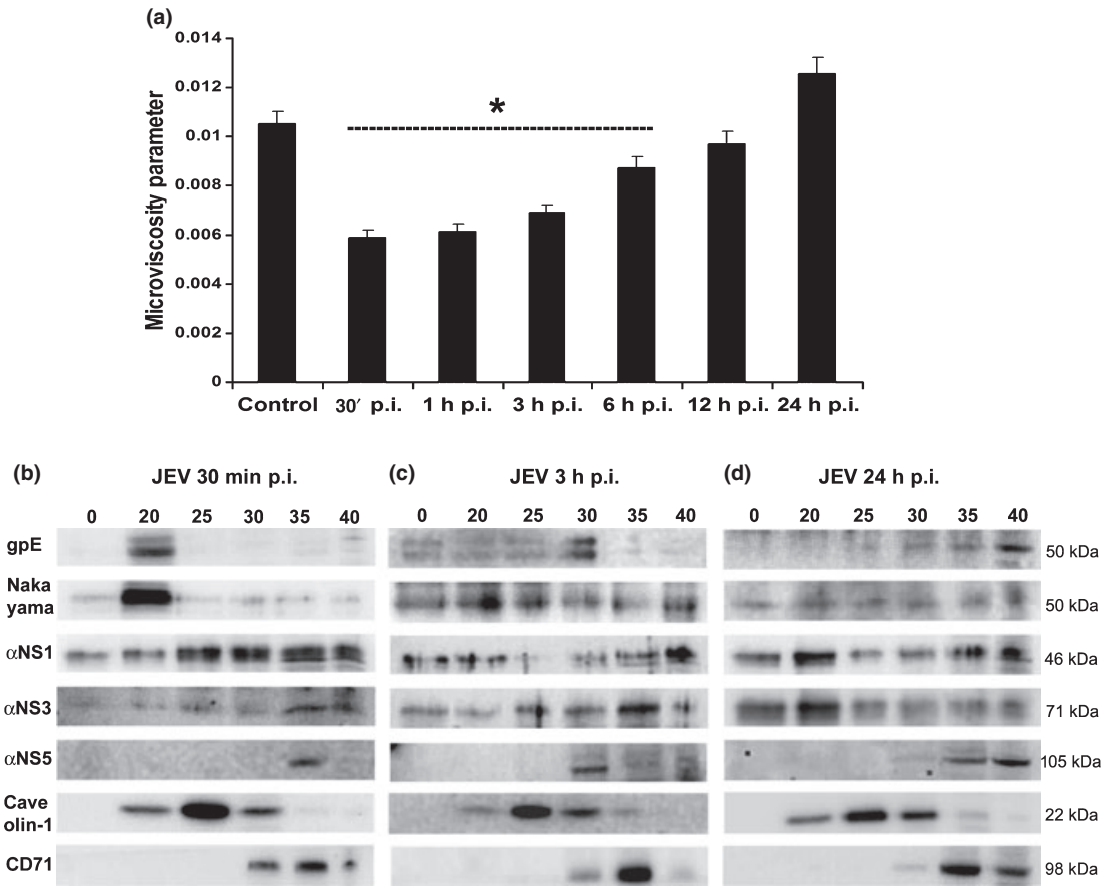

Fig. 2 Increase in membrane fluidity and role of lipid rafts during JEV entry and replication in C17.2 cells. Control and JEV infected C17.2 at different h p.i. were incubated with fluorescent membrane probe DPH and the microviscosity parameter was calculated (a). A significant decrease in microviscosity was observed as early as 30 min p.i. and was maintained until 6 h p.i. At later time points membrane microviscosity was restored back to control levels or even higher. Values represent average value ± SEM from triplicate experiments. * represents significant change compared to control, $p < 0.01$. Lipid rafts or DRMs were isolated from JEV infected C17.2 cells at 30 min p.i. (b), 3 h p.i. (c) and 24 h p.i. (d) using Optiprep density gradient centrifugation. Different membrane fractions were collected and the values (0–40) represent the percentage of Optiprep (lower value corresponds to lighter fractions). Western blotting for Caveolin-1 (raft marker) and CD71 (non-raft marker) show efficient separation of the different membrane fractions. JEV gpE and Nakayama (envelope protein) was localised primarily to the rafts at 30 min p.i. (b), but was distributed in all fractions at 3 h p.i. (c) and 24 h p.i. (d). While αNS1 and αNS3 were distributed in both raft and non-raft fractions at early time points of infection (30 min p.i. and 3 h p.i.), these non-structural proteins accumulated more in the lighter raft fractions at 24 h p.i. (d).

Localisation of JEV structural and non-structural proteins to lipid rafts

The increase in membrane fluidity implicates that membrane cholesterol and lipid rafts may play an important role in early infection stages. To test this hypothesis, membrane fractions were isolated from JEV infected C17.2 cells at different h p.i. by Opti-prep density gradient centrifugation. Western blotting with Caveolin-1 (raft marker), and CD71 (non-raft marker) showed efficient separation of low-density raft and higher-density non-raft fractions. At 30 min p.i., JEV gpE was completely clustered into the raft fractions, but at later time points (3 h and 24 h p.i.) was distributed throughout the membrane (Fig. 2b–d). Similar membrane distribution, with increased clustering in rafts during early infection (30 min p.i.) was observed with Nakayama antibody, which recognises envelope protein of all viruses belonging to JE serogroup. Thus, it was confirmed that the structural envelope protein indeed co-localises with the cholesterol-enriched raft domains as early as 30′ p.i., possibly aiding in virus entry into C17.2.

The non-structural proteins, however, show a different distribution pattern in the membrane fractions. The αNS1 protein which has a role in negative strand RNA synthesis is present in all the membrane fractions, but at 24 h p.i. show increased localisation to the lighter raft fractions (Fig. 2d). αNS3 and αNS5, both belonging to the viral replicase, however are distributed differentially in the membrane. Although, both these NS proteins are predominantly in the non-raft fractions at 30 min p.i., there is translocation to the caveolin-rich raft fractions (more prominent in case of NS3) at 3 h p.i. (Fig. 2b and c). Interestingly, at 24 h p.i., while αNS3 is concentrated mostly in rafts, αNS5 remains confined to the non-raft fractions (Fig. 2d). Thus, both NS1 and NS3, which are essential for JEV replication and not during entry *per se* were localised to the non-raft fractions at early time points, but cluster in raft fractions at later infection stages, possibly when viral replication occurs.

Lipid raft disruption by MBCD during JEV entry reduces viral load in C17.2

Since envelope proteins important for virus entry are localised in the rafts, we hypothesised that lipid rafts play key roles during JEV entry. One of the main components of lipid rafts is cholesterol, and we therefore investigated the effect of cholesterol depletion (using MBCD) on virus entry. MBCD is a derivative of cyclic oligosaccharides and has a lipophilic property that extracts cholesterol from membranes, resulting in lipid raft disruption. C17.2 were treated with MBCD (2.5–10 mM) for 1 h at 37°C, washed and incubated with fresh serum free media. 24 h later, MTS assay was performed to determine the percentage of cell survival (Figure S1a). The concentration of 5 mM has been used previously in other cell lines for cholesterol depletion studies (Lee *et al.* 2008) and we also selected this dose where approximately 75% cell survival was observed.

The approach with MBCD was: pre-treatment with MBCD for 1 h followed by JEV infection for 1 h (M + JEV) or post-treatment with MBCD for 1 h (JEV + M). The cells were then cultured in serum-free DMEM for 24 h p.i., and RNA was isolated and the supernatant were collected from the different conditions. Semi-quantitative RT-PCR for GP78 RNA showed a significant decrease in JEV RNA levels upon pre-treatment with MBCD (M + JEV), but not post-treatment (JEV + M) compared to JEV-infected cells (Figure S1b) ($p < 0.05$). Plaque assay performed using culture supernatant from C17.2 under above-mentioned conditions showed a twofold decrease in virus titre in supernatant from M + JEV condition compared to that from JEV infection alone (Figure S1c) ($p < 0.05$). This data clearly shows that disruption of rafts by cholesterol depletion immediately before JEV infection, leads to reduction in viral load and infective virus particle production, possibly because of initial decrease in JEV entry.

JEV internalisation is lipid raft dependent, but not caveolae-mediated

To further demonstrate that JEV entry is mediated by cholesterol-rich microdomains, we examined the internalisation of gpE and biotin-conjugated CTB by C17.2 cells. CTB by binding to its receptor GM1 enters cells in a lipid raft dependent manner (Fujinaga *et al.* 2003). C17.2 cells were incubated with 5 multiplicity of infection of JEV and 10 μg/mL CTB at 4°C for 45 min for attachment. Cells were then shifted to 37°C for 30 min to allow internalisation. Dual immunofluorescence show discrete areas of co-localisation of gpE with CTB on the plasma membrane in JEV infected cells (Fig. 3b), but not in control cells (Fig. 3a). This strongly suggests the involvement of lipid rafts for internalisation of JEV virions.

As caveolae form an integral part of membrane rafts and act as portals for virus entry (Shin and Abraham 2001), we wanted to check if the JEV entry into NSPCs occurs via caveolae-mediated endocytosis. Control and JEV infected C17.2 at 1 h p.i. were fixed and stained for gpE along with Caveolin-1. Immunocytochemistry for both JEV gpE and Caveolin-1 did not show any co-localisation at early time points of JEV infection (Fig. 3d). This indicated that JEV do not utilise the caveolae in the lipid rafts for entry into cells.

JEV internalisation occurs by clathrin-mediated endocytosis

We next examined whether clathrin-mediated endocytosis play any role in JEV internalisation. C17.2 cells were incubated with Alexa 594-labelled transferrin and/or JEV at 4°C for 45 min and then shifted to 37°C for 45 min to allow cellular uptake by endocytosis. Distinct co-localisation of JEV gpE with transferrin (the marker ligand for clathrin mediated endocytosis) was observed in the cytoplasm and

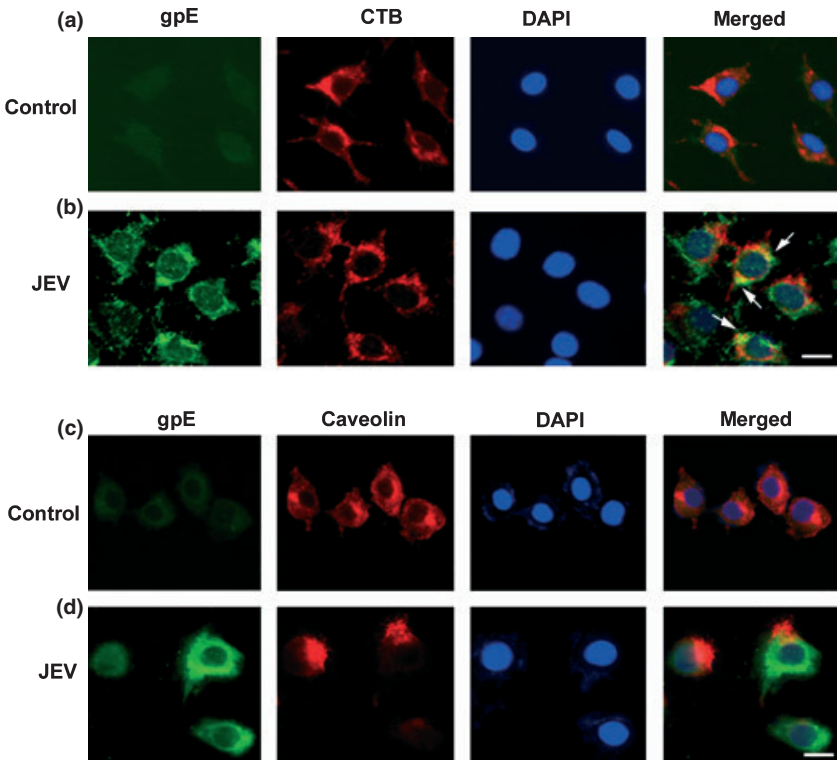

Fig. 3 JEV gpE co-localise with CTB, but not with caveolin at the plasma membrane. C17.2 cells grown in 8-well chamber slides were infected with JEV at 5 MOI and incubated with 10 μg/mL of biotin-conjugated CTB for 45 min at 4°C. Cells were shifted to 37°C for 30 min and then fixed with 2% PFA. Streptavidin-Alexa 594 (red) was added to detect CTB, followed by staining with JEV gpE (FITC, green). Both control (a) and infected cells (b) show CTB expression. Discrete areas of co-localisation of gpE with CTB were observed in JEV infected C17.2 (b), as shown by arrows. Double immunocytochemistry for gpE (FITC, green) and Caveolin-1 (Alexa 594, red) was performed on control and (c) and JEV infected cells (d). Cells were counter-stained with DAPI and visualised under 40× magnification of Zeiss Apotome microscope. No co-localisation of gpE with Caveolin-1 in JEV infected cells (d) was observed. Scale bar corresponds to 20 microns.

perinuclear areas of JEV infected cells (Fig. 4b), but not control cells (Fig. 4a).

To further confirm the specific role of clathrin-mediated endocytosis during JEV entry, C17.2 was transfected with double negative mutants of clathrin. Eps15 is a conserved protein necessary for formation of clathrin vesicles and organised into three domains: N-terminal domain (DI) which has three Eps-15-Homology (EH) domains involved in protein-protein interactions, central domain (DII) and C-terminal domain (DIII). A double negative mutant of Eps15 (GFP-EΔ95/295), lacking the second and third Eps-15-Homology domains was used for the study as it interferes with clathrin-coated vesicle formation (Benmerah *et al.* 1999). Another construct GFP-D3Δ2, which does not interfere with clathrin-mediated uptake, was used as a control (both constructs a kind gift from Prof. Benmerah, France). C17.2 cells were transiently transfected with the constructs using Lipofectamine 2000, and 24 h post-transfection, cells were infected with JEV for 1 h, and at 6 h p.i. stained with gpE. Interestingly in GFP-EΔ95/295 transfected cells, gpE accumulate and disperse in patches mostly on the cell surface and fail to be internalised (Fig. 4d). In D3Δ2 transfected ones, efficient JEV uptake occurs and gpE was localised in cytoplasm and in the perinuclear areas (Fig. 4c). These results together show that though JEV associates with membrane rafts, the entry of the virus into C17.2 occurs via clathrin-mediated endocytosis.

Association of Src tyrosine kinases with lipid rafts in early stages of JEV infection

Previous reports have shown that JEV infection at early stages stimulates hyperphosphorylation of Src family of non-receptor tyrosine kinases (Raung *et al.* 2007). As these kinases are membrane bound (Mukherjee *et al.* 2003; Vacaresse *et al.* 2008), we investigated whether JEV infection alters the localisation of these phosphorylated kinases between rafts and non-rafts. Membrane fractions isolated by Opti-prep density gradient centrifugation from control and JEV infected (30 min p.i. and 3 h p.i.) C17.2 were western blotted for phospho c-Src kinase (p-Src, Tyr416), phospho-Hck (pHck, Tyr209/Ser211), and phospho-FAK (p-FAK,

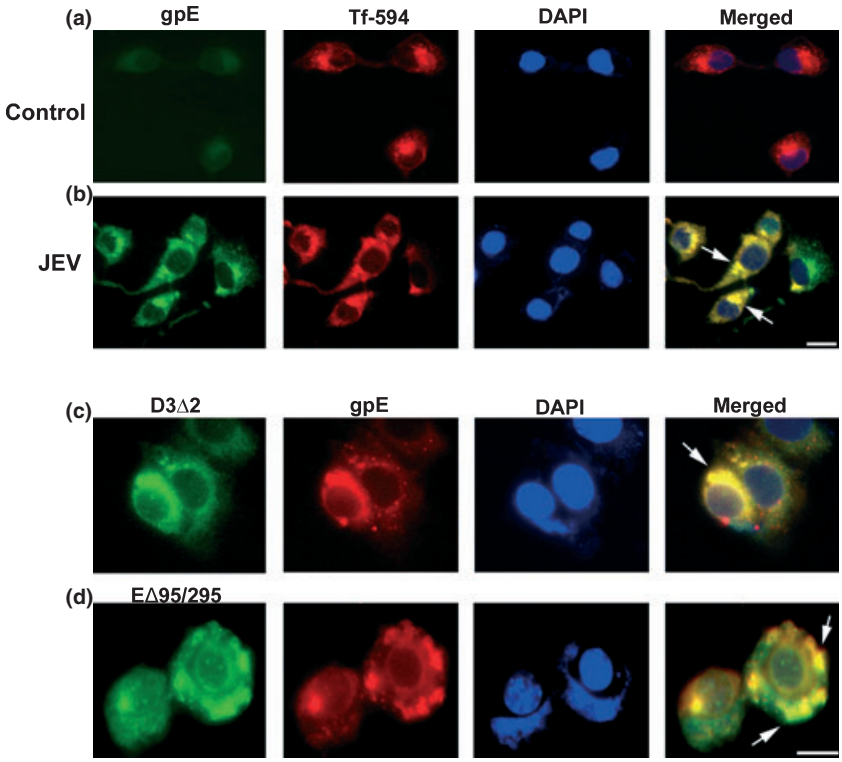

Fig. 4 JEV gpE internalise into C17.2 via clathrin-mediated endocytosis. C17.2 cells grown in 8-well chamber slides were incubated with Alexa 594-labelled transferrin (Tf-594, red) in control (a) and JEV infected (b) for 45 min at 4°C. Cells were shifted to 37°C for 45 min and then fixed in 4% PFA. Cells were stained with gpE (FITC, green) and counter-stained with DAPI. Tf-594, which enters specifically by clathrin-mediated endocytosis completely co-localises with gpE in JEV infected cells (b), as shown by arrows. C17.2 cells grown on 4-well chamber slides were transfected with two GFP-tagged plasmid constructs: a dominant negative mutant of clathrin (GFP-EΔ95/295) and the other where clathrin-mediated endocytosis is not hampered (GFP-D3Δ2). 24 h post-transfection, the cells were infected with JEV and then at 6 h p.i. immunocytochemistry for gpE (Alexa 594, red) was performed. Cells were counter-stained with DAPI and images were captured under 40× magnification. In GFP-D3Δ2 transfected cells, gpE was distributed in the cytoplasm and in the perinuclear regions (shown by arrows), and co-localisation with the mutant clathrin was also observed (c). However, in EΔ95/295 transfected cells, gpE along with the double negative clathrin mutant remained mostly confined to the cell surface and was not internalised (shown by arrows) (d). Scale bar corresponds to 20 microns.

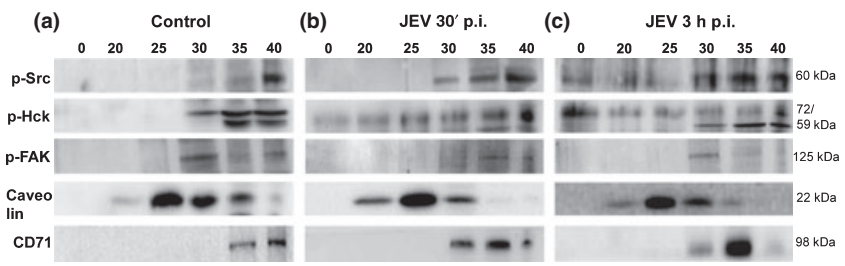

Fig. 5 Association of Src tyrosine kinases with lipid rafts in early stages of JEV infection in C17.2. Lipid rafts/DRMs were isolated from control (a) and JEV infected cells at 30 min p.i.(b) and 3 h p.i.(c). Efficient separation of rafts from non-rafts was seen with Caveolin-1 and CD71. Western blotting for p-Src (Tyr416), p-Hck (Tyr209/Ser211) and p-FAK (Tyr397) showed localisation of these proteins mostly on the non-raft fractions in control cells (a). At 30 min p.i., p-Src and p-Hck translocated to the lighter membrane fractions (b), which was more evident at 3 h p.i. (c). However, p-FAK remained mostly confined to the non-raft fractions at both the time points of infection.

Tyr397). While p-Src was localised to non-raft fractions in control cells, it translocated to raft fractions at 30 min p.i (Fig. 5a and b). The co-localisation of p-Src with lighter raft fractions was more prominent at 3 h p.i (Fig. 5c). p-Hck localised predominantly in non-rafts in control cells, but at both 30 min and 3 h p.i greater association with the lighter

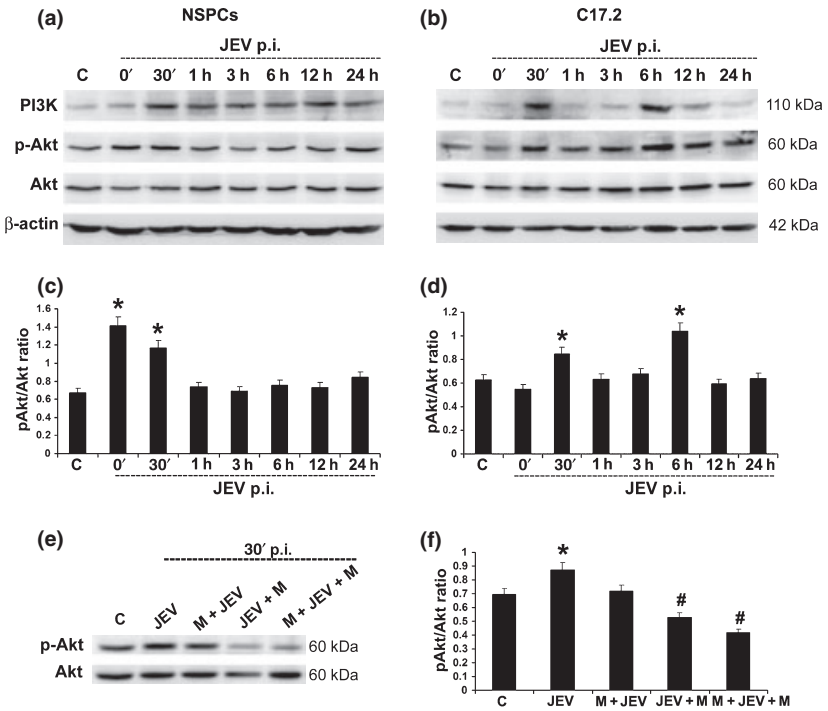

Fig. 6 Activation of PI3K/Akt signalling in early JEV infection in C17.2 and its dependence on intact lipid rafts. Cell lysates were prepared from control and JEV infected neurospheres/NSPCs (a) and C17.2 (b) at different time points of infection and western blotting was performed. An increase in PI3K (p110α) was observed in both NSPCs and C17.2 cells as early as 30 min p.i. The graphs represent the ratio of p-Akt/Akt, which indicates the extent of phosphorylation of Akt (c,d). In NSPCs, significant increase in Akt phosphorylation was observed as early as 0 min and 30 min p.i. (c). In C17.2 cells, Akt phosphorylation showed a biphasic response, a significant increase at 30 min p.i., followed by a decrease and again an increase at 6 h p.i. (d). C17.2 cells were either uninfected (C), or JEV infected (JEV) or treated with MBCD under three different approaches: pre-treatment with MBCD for 30 min followed by JEV infection (M + JEV), or JEV infection followed by MBCD treatment for 30 min (JEV + M) or MBCD pre-treatment followed by JEV infection and then post-treatment with MBCD for 30 min (M + JEV + M). Cells were collected at 30 min p.i. and protein was isolated. Western blot showed an increase in p-Akt in JEV infection at 30 min p.i., and a dramatic decrease in JEV + M and M + JEV + M conditions (e). The graph showing pAkt/Akt ratio indicates significant decrease in phosphorylation of Akt in JEV + M and M + JEV + M (f), suggesting that lipid rafts disruption post-JEV infection impairs PI3K/Akt signalling. Values represent average value ± SEM from three independent experiments. * represents significant change from control, $p < 0.01$; # represents significant change from JEV, $p < 0.01$.

raft fractions was observed. In contrast, p-FAK which was associated with both raft and non-raft portions in control, was confined mostly to non-raft fractions upon JEV infection and no distinct change in its distribution between the membrane fractions was observed (Fig. 5a–c). These findings indicated that p-Src and p-Hck, associate with raft membranes during early JEV infection stages, and possibly triggers downstream signalling.

Activation of PI3K/Akt signalling in early JEV infection is dependent on lipid rafts

As SFKs have been shown to be associated with PI3K class IA p85/p110α in lipid rafts, and activate PI3K/Akt signalling (Vanhaesebroeck *et al*. 2010), we investigated whether localisation of Src kinases to rafts in early infection activate PI3K signalling in C17.2 and NSPCs. Indeed, cell lysates from both NSPCs and C17.2 showed elevated levels of p110α-PI3K at 30 min p.i. (Fig. 6a and b). While in NSPCs, the increase in PI3K was sustained over the entire infection period, however, in C17.2 cells, a biphasic pattern of increase was observed. The phosphorylation of Akt (indicated by pAkt/Akt ratio) showed a significant increase in NSPCs as early as 0 min and 30 min p.i., but not at later time points (Fig. 6c). Interestingly, in C17.2 cells, Akt phosphorylation showed increase at 30 min and 6 h p.i compared to control cells, a similar biphasic pattern of increase as observed in case of PI3K expression (Fig. 6d) ($p < 0.01$).

To further validate that lipid rafts are critical in triggering PI3K/Akt signalling during early JEV infection, membrane cholesterol was depleted in C17.2 cells using MBCD; 30 min prior to infection (M + JEV), 30 min post-infection (JEV + M), and both pre- and post-infection (M + JEV + M). Interestingly, a dramatic decrease in Akt phosphorylation was observed in JEV + M and M + JEV + M conditions compared to JEV infected ($p < 0.01$) (Fig. 6e and f), clearly

indicating that disruption of lipid rafts immediately after JEV infection hamper PI3K/Akt signalling in the infected cells.

Discussion

In this study, we found that lipid rafts play a critical role in JEV entry into neural stem cells (C17.2 cells) in at least two ways: (i) association of the virus envelope proteins with the lipid rafts (which possibly helps to concentrate the virus receptors present on the host cell membrane) and (ii) activation of SFKs and downstream PI3K/Akt pathway which maintains host cell survival in initial stages of infection. Interestingly, we also found that though JEV utilises the rafts, however, internalisation into C17.2 occurs by clathrin-mediated endocytosis and do not involve caveolae which are mostly associated with the rafts. The importance of lipid rafts in other stages of the JEV life cycle was observed, specially during virus replication as shown by the association of different components of the replication complex (NS1 and NS3) with the rafts at later time points of infection (24 h p.i.).

Dynamic membrane rearrangements have been shown to occur during various stages of viral infections, from initial virus-cell encounter to release of virus particles (Chazal and Gerlier 2003; Nayak and Hui 2004; Suzuki and Suzuki 2006). We also observed that following JEV infection in C17.2, significant increase in the membrane fluidity occurred at initial stages until 6 h p.i. The increase in lipid fluidity is an early event induced by adsorption of virions on the host surface (Levanon *et al.* 1977). A direct consequence of changes in lipid fluidity would be alterations in the lipid raft arrangements in the host cell membrane.

The formation of lipid rafts arises from the tight packing of cholesterol with saturated sphingolipid chains that allows for increased order in the membrane and resistance to non-ionic detergents at 4°C (Brown and London 1998; Glebov and Nichols 2004). Lipid rafts are important for a number of cellular processes, besides acting as portals for virus entry (Manes *et al.* 2003). Most enveloped viruses have evolved to utilise lipid rafts or cholesterol to support their entry and replication in host cells, like influenza, measles, vesicular stomatitis virus, Simian foamy virus, Sindbis virus, and Retrovirus like HIV (Chazal and Gerlier 2003; Suzuki and Suzuki 2006). We also observe the localisation of envelope proteins (gpE and Nakayama) to the raft membrane fractions at initial time points of JEV infection (30 min p.i. and 3 h p.i.). Considering that the Flaviviral gpE binds to host cellular receptors and carries out mixing of the virus and cellular membranes (Perera *et al.* 2008), co-localisation studies of JEV gpE with CTB confirmed that indeed JEV entry into cells is lipid-raft dependent. It has been shown that lipid rafts help to cluster virus receptors, for example, Hsp70 and Hsp90 (the putative receptors for DENV) have been shown to be localised in the rafts (Reyes-Del Valle *et al.* 2005). Though the specific cellular receptors for JEV internalisation have not yet been identified, we speculate that these receptors maybe present on the raft fractions, which possibly explains the observed localisation of envelope proteins with cholesterol enriched rafts.

Flavivirus entry mostly occurs via receptor-mediated endocytosis which is clathrin-dependent, as reported in case of WNV, DENV-2, and JEV (Nawa *et al.* 2003; Chu and Ng 2004; Acosta *et al.* 2008). Recent reports indicate a number of non-clathrin-mediated endocytic routes utilized by viruses, mostly caveolae-dependent (Shin and Abraham 2001). As caveolae are integral parts of lipid rafts (Glebov and Nichols 2004), and JEV gpE associates with rafts, therefore, a possibility of caveolae-mediated internalisation of JEV into C17.2 exists. However, we do not observe any co-localisation of JEV gpE with Caveolin-1 at 1 h p.i., indicating that perhaps caveolae do not play any role during entry. On the other hand, complete co-localisation of gpE with transferrin (which is trafficked by clathrin-mediated endocytosis) was observed. Furthermore, transfection of C17.2 with a dominant negative clathrin mutant lead to failure of JEV gpE internalisation into the cytoplasm. From these observations we propose that once JEV associates with the receptors (unidentified until now) on the lipid rafts, the virus enters these stem cells by clathrin-mediated endocytosis.

Besides entry, we demonstrate that the membrane rafts play critical role during JEV replication in these stem cells. The JEV replication complex consists of seven NS proteins, of which NS1, NS3 and NS5 has important functions (Uchil and Satchidanandam 2003). While NS3 is a multifunctional enzyme, NS5, the largest of all viral proteins is the RNA-dependent RNA polymerase. NS1 helps in synthesis of negative strand viral RNA. The distribution of these NS proteins on the membrane fractions presented an interesting feature: all of them remained confined to the non-raft fractions during initial infection stages, and at later time point (24 h p.i.) NS1 and NS3 moved to the lighter raft fractions. The association of NS1 and NS3 with lipid rafts corroborate previous findings that these rafts may serve to concentrate the various components of the replication complex as well as protect the newly synthesized viral RNA (Westaway *et al.* 2003; Salonen *et al.* 2005). Recently, it has been shown that the NS proteins of both DENV-2 and JEV localise to the lipid rafts/DRMs of infected cell membranes, the possible sites of Flaviviral replication (Lee *et al.* 2008; Noisakran *et al.* 2008).

It is well-documented that lipid rafts act as signalling platforms and initiate a variety of signal transduction processes. Previous reports have documented hyperphosphorylation of Src tyrosine kinase in JEV infection and therefore we investigated how the SFKs are distributed in the different membrane fractions isolated from control and JEV infected C17.2 (30 min and 3 h p.i.). Full activation of c-Src requires phosphorylation at Tyr416, which then allows

p-Src to associate with inner leaflet of the membrane (Brown and Cooper 1996; de Diesbach *et al.* 2008). While in control cells, both p-Src and p-Hck remain mostly in non-raft fractions, however, at early time point of JEV infection (30 min and 3 h p.i.) distribute to the raft fractions. The SFKs can localise to rafts by virtue of lipid modification and once associated physically interacts with a number of substrates including non-receptor tyrosine kinases like FAK and phosphorylate them (Brown and Cooper 1996; Thomas and Brugge 1997). In our studies, we did not observe any change in the localisation of p-FAK (Tyr397) between raft and non-raft fractions in control and JEV infected cells. FAK localisation into lipid microdomains is induced by integrin engagement (Baillat *et al.* 2008), however, integrins and integrin-dependent FAK signalling are not critical during Flavivirus entry (Medigeshi *et al.* 2008), which possibly explains our findings of non-association of FAKs with lipid rafts.

Increasing evidence have shown the association of SFKs with PI3K class IA p85/p110α in lipid rafts resulting in subsequent activation of PI3K/Akt signalling (Vanhaesebroeck *et al*. 2010). Western blot analysis using cell lysates from control and JEV infected C17.2 and NSPCs show the increase in PI3K p110α, the catalytic subunit of PI3K. Increased Akt phosphorylation was also observed in both the cell line and primary cultures at early time points of JEV infection. In C17.2 cells, we however observe a biphasic pattern of PI3K/Akt activation. Such biphasic activity of class IA-PI3K has been reported following receptor stimulation (Stein and Waterfield 2000) as well as in early stages of virus infections (Yu and Alwine 2002; Ehrhardt *et al.* 2007). It has been proposed that early peaking of PI3K is mediated by virus binding to host surface, and later activity is associated with generation of anti-apoptotic signals (Ehrhardt *et al.* 2007). In Flaviviral infections, the importance of PI3K/Akt pathway in maintaining host cell survival and stimulating anti-apoptotic pathways during early infection has been established (Cooray 2004; Lee *et al.* 2005). Besides, the role of PI3K/Akt pathway in virus entry and replication has also been implicated (Saeed *et al.* 2008).

The most common approach to manipulate rafts is to alter the cholesterol levels, and this influences the cell's ability to be infected by the virus. In Flaviviral infections, contradictory reports on the role of cholesterol in different stages of virus life cycle exits. While cholesterol is required for later replication steps, addition of cholesterol readily during virus adsorption has been shown to block the entry process (Lee *et al.* 2008). However, most reports suggest a significant role of cholesterol both during entry and replication (Rawat *et al.* 2003). Our data from C17.2 shows that disruption of the lipid rafts using cholesterol-depleting agents like MBCD prior to virus treatment, results in decreased levels of cellular JEV RNA as well less production of infectious virus particles (as shown by plaque assay). This effect was not significant when rafts were disrupted following JEV adsorption, suggesting that intact lipid rafts are essential during the initial virus adsorption and entry stages. However, cholesterol depletion did not decrease JEV RNA load many fold, which can be explained by the classical notion that viruses which utilise clathrin-mediated endocytosis are less sensitive to cholesterol depletion than viruses which utilise mostly caveolar pathway (Vela *et al.* 2007). We further observed that disruption of lipid rafts post-JEV infection impairs the PI3K/Akt signalling and results in dramatic decline in Akt phosphorylation during early infection. This established the critical role of lipid rafts in initiation of signalling cascades during early JEV infection.

From all the above findings and discussion, we have been able to clearly establish the importance of the lipid rafts in the JEV life cycle in neural stem cells as well as in triggering Src and PI3K/Akt signalling in order to maintain the host cell survival during early infection. Further research into how the association with the lipid rafts facilitates JEV entry, specifically interaction of JEV gpE with the putative virus receptors on the lipid rafts needs to be carried out.

Acknowledgements

The authors acknowledge Ms. Richa Tewari for help in isolation of lipid rafts. Technical assistance from Kanhaiya Lal Kumawat and grant support from Dept. of Biotechnology, Govt. of India (# BT/PR8682/Med/14/1273/2007) awarded to AB is also highly appreciated. SD is a recipient of Senior Research Fellowship from University Grants Commission. The authors would like to thank Dr Guruprasad Medigeshi from Vaccine and Infectious Diseases Research Center, THSTI, Gurgaon for critical comments on the work.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Cholesterol depletion reduces the viral load and production of infectious virus particles in C17.2 cells.

As a service to our authors and readers, this journal provides supporting information supplied by the authors. Such materials are peer-reviewed and may be re-organized for online delivery, but are not copy-edited or typeset. Technical support issues arising from supporting information (other than missing files) should be addressed to the authors.

References

Acosta E. G., Castilla V. and Damonte E. B. (2008) Functional entry of dengue virus into Aedes albopictus mosquito cells is dependent on clathrin-mediated endocytosis. *J. Gen. Virol.* **89**, 474–484.

Anderson R. (2003) Manipulation of cell surface macromolecules by flaviviruses. *Adv. Virus Res.* **59**, 229–274.

Baillat G., Siret C., Delamarre E. and Luis J. (2008) Early adhesion induces interaction of FAK and Fyn in lipid domains and activates raft-dependent Akt signaling in SW480 colon cancer cells. *Biochim. Biophys. Acta* **1783**, 2323–2331.

Benmerah A., Bayrou M., Cerf-Bensussan N. and Dautry-Varsat A. (1999) Inhibition of clathrin-coated pit assembly by an Eps15 mutant. *J. Cell Sci.* **112**(Pt 9), 1303–1311.

Brown D. A. (2006) Lipid rafts, detergent-resistant membranes, and raft targeting signals. *Physiology (Bethesda)* **21**, 430–439.

Brown M. T. and Cooper J. A. (1996) Regulation, substrates and functions of src. *Biochim. Biophys. Acta* **1287**, 121–149.

Brown D. A. and London E. (1998) Functions of lipid rafts in biological membranes. *Annu. Rev. Cell Dev. Biol.* **14**, 111–136.

Chazal N. and Gerlier D. (2003) Virus entry, assembly, budding, and membrane rafts. *Microbiol. Mol. Biol. Rev.* **67**, 226–237.

Chu J. J. and Ng M. L. (2004) Infectious entry of West Nile virus occurs through a clathrin-mediated endocytic pathway. *J. Virol.* **78**, 10543–10555.

Cooray S. (2004) The pivotal role of phosphatidylinositol 3-kinase-Akt signal transduction in virus survival. *J. Gen. Virol.* **85**, 1065–1076.

Das S. and Basu A. (2008) Japanese encephalitis virus infects neural progenitor cells and decreases their proliferation. *J. Neurochem.* **106**, 1624–1636.

Das S., Ghosh D. and Basu A. (2009) Japanese encephalitis virus induce immuno-competency in neural stem/progenitor cells. *PLoS ONE* **4**, e8134.

Diagana M., Preux P. M. and Dumas M. (2007) Japanese encephalitis revisited. *J. Neurol. Sci.* **262**, 165–170.

de Diesbach P., Medts T., Carpentier S. *et al.*(2008) Differential subcellular membrane recruitment of Src may specify its downstream signalling. *Exp. Cell Res.* **314**, 1465–1479.

Ehrhardt C., Wolff T., Pleschka S., Planz O., Beermann W., Bode J. G., Schmolke M. and Ludwig S. (2007) Influenza A virus NS1 protein activates the PI3K/Akt pathway to mediate antiapoptotic signaling responses. *J. Virol.* **81**, 3058–3067.

Fujinaga Y., Wolf A. A., Rodighiero C., Wheeler H., Tsai B., Allen L., Jobling M. G., Rapoport T., Holmes R. K. and Lencer W. I. (2003) Gangliosides that associate with lipid rafts mediate transport of cholera and related toxins from the plasma membrane to endoplasmic reticulm. *Mol. Biol. Cell* **14**, 4783–4793.

Ghosh D. and Basu A. (2009) Japanese encephalitis-a pathological and clinical perspective. *PLoS Negl. Trop. Dis.* **3**, e437.

Ghoshal A., Das S., Ghosh S., Mishra M. K., Sharma V., Koli P., Sen E. and Basu A. (2007) Proinflammatory mediators released by activated microglia induces neuronal death in Japanese encephalitis. *Glia* **55**, 483–496.

Glebov O. O. and Nichols B. J. (2004) Distribution of lipid raft markers in live cells. *Biochem. Soc. Trans.* **32**, 673–675.

Golub T., Wacha S. and Caroni P. (2004) Spatial and temporal control of signaling through lipid rafts. *Curr. Opin. Neurobiol.* **14**, 542–550.

Lee C. J., Liao C. L. and Lin Y. L. (2005) Flavivirus activates phosphatidylinositol 3-kinase signaling to block caspase-dependent apoptotic cell death at the early stage of virus infection. *J. Virol.* **79**, 8388–8399.

Lee C. J., Lin H. R., Liao C. L. and Lin Y. L. (2008) Cholesterol effectively blocks entry of flavivirus. *J. Virol.* **82**, 6470–6480.

Levanon A., Kohn A. and Inbar M. (1977) Increase in lipid fluidity of cellular membranes induced by adsorption of RNA and DNA virions. *J. Virol.* **22**, 353–360.

Mackenzie J. S., Johansen C. A., Ritchie S. A., van den Hurk A. F. and Hall R. A. (2002) Japanese encephalitis as an emerging virus: the emergence and spread of Japanese encephalitis virus in Australasia. *Curr. Top. Microbiol. Immunol.* **267**, 49–73.

Mackenzie J. M., Khromykh A. A. and Parton R. G. (2007) Cholesterol manipulation by West Nile virus perturbs the cellular immune response. *Cell Host Microbe* **2**, 229–239.

Manes S., del Real G. and Martinez A. C. (2003) Pathogens: raft hijackers. *Nat. Rev. Immunol.* **3**, 557–568.

Medigeshi G. R., Hirsch A. J., Streblow D. N., Nikolich-Zugich J. and Nelson J. A. (2008) West Nile virus entry requires cholesterol-rich membrane microdomains and is independent of alphavbeta3 integrin. *J. Virol.* **82**, 5212–5219.

Mukherjee A., Arnaud L. and Cooper J. A. (2003) Lipid-dependent recruitment of neuronal Src to lipid rafts in the brain. *J. Biol. Chem.* **278**, 40806–40814.

Myint K. S., Gibbons R. V., Perng G. C. and Solomon T. (2007) Unravelling the neuropathogenesis of Japanese encephalitis. *Trans. R. Soc. Trop. Med. Hyg.* **101**, 955–956.

Nawa M., Takasaki T., Yamada K., Kurane I. and Akatsuka T. (2003) Interference in Japanese encephalitis virus infection of Vero cells by a cationic amphiphilic drug, chlorpromazine. *J. Gen. Virol.* **84**, 1737–1741.

Nayak D. P. and Hui E. K. (2004) The role of lipid microdomains in virus biology. *Subcell. Biochem.* **37**, 443–491.

Noisakran S., Dechtawewat T., Avirutnan P., Kinoshita T., Siripanyaphinyo U., Puttikhunt C., Kasinrerk W., Malasit P. and Sittisombut N. (2008) Association of dengue virus NS1 protein with lipid rafts. *J. Gen. Virol.* **89**, 2492–2500.

Perera R., Khaliq M. and Kuhn R. J. (2008) Closing the door on flaviviruses: entry as a target for antiviral drug design. *Antiviral Res.* **80**, 11–22.

Raung S. L., Chen S. Y., Liao S. L., Chen J. H. and Chen C. J. (2007) Japanese encephalitis virus infection stimulates Src tyrosine kinase in neuron/glia. *Neurosci. Lett.* **419**, 263–268.

Rawat S. S., Viard M., Gallo S. A., Rein A., Blumenthal R. and Puri A. (2003) Modulation of entry of enveloped viruses by cholesterol and sphingolipids (Review). *Mol. Membr. Biol.* **20**, 243–254.

Reyes-Del Valle J., Chavez-Salinas S., Medina F. and Del Angel R. M. (2005) Heat shock protein 90 and heat shock protein 70 are components of dengue virus receptor complex in human cells. *J. Virol.* **79**, 4557–4567.

Saeed M. F., Kolokoltsov A. A., Freiberg A. N., Holbrook M. R. and Davey R. A. (2008) Phosphoinositide-3 kinase-Akt pathway controls cellular entry of Ebola virus. *PLoS Pathog.* **4**, e1000141.

Salonen A., Ahola T. and Kaariainen L. (2005) Viral RNA replication in association with cellular membranes. *Curr. Top. Microbiol. Immunol.* **285**, 139–173.

Sharma V., Joseph C., Ghosh S., Agarwal A., Mishra M. K. and Sen E. (2007) Kaempferol induces apoptosis in glioblastoma cells through oxidative stress. *Mol. Cancer Ther.* **6**, 2544–2553.

Shin J. S. and Abraham S. N. (2001) Caveolae as portals of entry for microbes. *Microbes Infect.* **3**, 755–761.

Simons K. and Toomre D. (2000) Lipid rafts and signal transduction. *Nat. Rev. Mol. Cell Biol.* **1**, 31–39.

Snyder E. Y., Deitcher D. L., Walsh C., Arnold-Aldea S., Hartwieg E. A. and Cepko C. L. (1992) Multipotent neural cell lines can engraft and participate in development of mouse cerebellum. *Cell* **68**, 33–51.

Stein R. C. and Waterfield M. D. (2000) PI3-kinase inhibition: a target for drug development? *Mol. Med. Today* **6**, 347–357.

Stiasny K., Koessl C. and Heinz F. X. (2003) Involvement of lipids in different steps of the flavivirus fusion mechanism. *J. Virol.* **77**, 7856–7862.

Suzuki T. and Suzuki Y. (2006) Virus infection and lipid rafts. *Biol. Pharm. Bull.* **29**, 1538–1541.

Swarup V., Das S., Ghosh S. and Basu A. (2007) Tumor necrosis factor receptor-1-induced neuronal death by TRADD contributes to the

pathogenesis of Japanese encephalitis. *J. Neurochem.* **103**, 771–783.

Tewari R., Sharma V., Koul N. and Sen E. (2008) Involvement of miltefosine-mediated ERK activation in glioma cell apoptosis through Fas regulation. *J. Neurochem.* **107**, 616–627.

Thomas S. M. and Brugge J. S. (1997) Cellular functions regulated by Src family kinases. *Annu. Rev. Cell Dev. Biol.* **13**, 513–609.

Uchil P. D. and Satchidanandam V. (2003) Architecture of the flaviviral replication complex. Protease, nuclease, and detergents reveal encasement within double-layered membrane compartments. *J. Biol. Chem.* **278**, 24388–24398.

Vacaresse N., Moller B., Danielsen E. M., Okada M. and Sap J. (2008) Activation of c-Src and Fyn kinases by protein-tyrosine phosphatase RPTPalpha is substrate-specific and compatible with lipid raft localization. *J. Biol. Chem.* **283**, 35815–35824.

Vanhaesebroeck B., Guillermet-Guibert J., Graupera M. and Bilanges B. (2010) The emerging mechanisms of isoform-specific PI3K signalling. *Nat. Rev. Mol. Cell Biol.* **11**, 329–341.

Vela E. M., Zhang L., Colpitts T. M., Davey R. A. and Aronson J. F. (2007) Arenavirus entry occurs through a cholesterol-dependent, non-caveolar, clathrin-mediated endocytic mechanism. *Virology* **369**, 1–11.

Vrati S., Agarwal V., Malik P., Wani S. A. and Saini M. (1999) Molecular characterization of an Indian isolate of Japanese encephalitis virus that shows an extended lag phase during growth. *J. Gen. Virol.* **80**(Pt 7), 1665–1671.

Westaway E. G., Mackenzie J. M. and Khromykh A. A. (2003) Kunjin RNA replication and applications of Kunjin replicons. *Adv. Virus Res.* **59**, 99–140.

Yeatman T. J. (2004) A renaissance for SRC. *Nat. Rev. Cancer* **4**, 470–480.

Yu Y. and Alwine J. C. (2002) Human cytomegalovirus major immediate-early proteins and simian virus 40 large T antigen can inhibit apoptosis through activation of the phosphatidylinositide 3′-OH kinase pathway and the cellular kinase Akt. *J. Virol.* **76**, 3731–3738.

