Virus Research

Two PDZ binding motifs within NS5 have roles in Tick-borne encephalitis virus replication

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ABSTRACT

The flavivirus genus includes important human neurotropic pathogens like Tick-borne encephalitis virus (TBEV) and West-Nile virus (WNV). Flavivirus replication occurs at replication complexes, where the NS5 protein provides both RNA cap methyltransferase and RNA-dependent RNA polymerase activities. TBEVNS5 contains two PDZ binding motifs (PBMs) important for specific targeting of human PDZ proteins including Scribble, an association important for viral down regulation of cellular defense systems and neurite outgrowth.

To determine whether the PBMs of TBEVNS5 affects virus replication we constructed a DNA based sub-genomic TBEV replicon expressing firefly luciferase. The PBMs within NS5 were mutated individually and in concert and the replicons were assayed in cell culture. Our results show that the replication rate was impaired in all mutants, which indicates that PDZ dependent host interactions influence TBEV replication. We also find that the C-terminal PBMs present in TBEVNS5 and WNVNS5 are targeting various human PDZ domain proteins. TBEVNS5 has affinity to Zonula occludens-2 (ZO-2), GIAP C-terminus interacting protein (GIPC), calcium/calmodulin-dependent serine protein kinase (CASK), glutamate receptor inter-acting protein 2, (GRIP2) and Interleukin 16 (IL-16). A different pattern was observed for WNVNS5 as it associate with a broader repertoire of putative host PDZ proteins.

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1. Introduction

Tick-borne encephalitis virus (TBEV) and West-Nile virus (WNV) are both arthropod-borne flaviviruses that have a major impact on global health. TBEV causes a febrile illness that commonly progress into encephalitis with mortality rates as high as 20–30% (Gritsun et al., 2003; Mandl, 2005; Pletnev and Men, 1998). The WNV is a milder neurotropic flavivirus transmitted by *Culex* mosquitoes, which was the subject of much attention in 1999 when the virus switched continent and a more virulent virus appeared in the US (Hayes, 2001).

Flaviviruses possess a positive sense RNA genome that encodes a single polyprotein, which is co- and posttranslationally processed into three structural and seven non-structural (NS) proteins in the order C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5 (Chambers et al., 1990; Gritsun et al., 2003; Mandl, 2005). Flavivirus replication occurs at replication complexes (RCs) present within induced invaginations at the ER membrane (Miller et al., 2007; Overby et al., 2010). The RCs includes most of the NS proteins (Kapoor et al., 1995; Li et al., 1999; Mackenzie et al., 1999), where the NS5 protein provides the RNA cap methyltransferase (MTase) and RNA-dependent RNA polymerase (RdRp) activities coupled to the N-terminal and C-terminal domains, respectively (Ackermann and Padmanabhan, 2001; Egloff et al., 2002; Koonin, 1993; Malet et al., 2007; Yap et al., 2007). Each of these enzymatic properties is essential for viral replication. Even though flavivirus replication occurs in the cytoplasm (Li et al., 1999; Mackenzie et al., 1999), the dengue virus and the yellow fever virus NS5 proteins have been found to local-ize predominantly within the host-cell nucleus by utilizing the nuclear import machinery (Brooks et al., 2002; Buckley et al., 1992; Johansson et al., 2001; Kapoor et al., 1995; Pryor et al., 2007).

PDZ (PSD-95/Dlg/ZO-1) domains are protein-interaction mod-ules involved in maintaining cell polarity and regulation of synaptic dynamics (Hung and Sheng, 2002; Roche et al., 2002). Class 1 PDZ domains typically recognize proteins that carry the PDZ binding motif (PBM) X-(S/T)-X-ϕ (where X is any amino acid and ϕ is a hydrophobic residue, usually V, I or L) in their C-terminus (Harris and Lim, 2001), but internal PBMs have also been identified (Hillier et al., 1999; Penkert et al., 2004). Interestingly, small differences
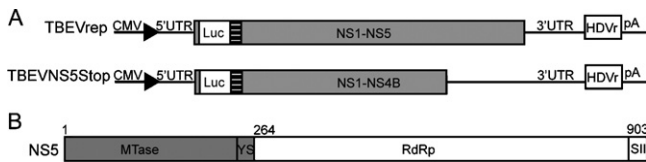
Fig. 1. Construction of DNA based subgenomic replicon models with mutations in the PBMs of NS5. (A) Schematic representation of the TBEVrep based on a cloned W-TBEV as described in material and methods. In the replicons, most of the sequence encoding the structural genes were removed and replaced with sequences encoding the GFP reporter gene and the autoprotease of FMDV (striped box). The constitutive promoter/enhancer of CMV, the HDVr ribozyme and the SV40 polyadenylation signal (pA) are indicated. TBEVNS5Stop contains two stop codons terminating translation after NS4B. (B) Schematic representation of TBEV NS5 with indicated domains methyltransferase (MTase) and RNA-dependent RNA polymerase (RdRp). The C-terminal and internal PBMs are indicated as SII and YS, respectively.

within the PBM of the influenza A virus (IAV) NS1 protein has been highlighted as a virulence determinant (Obenauer et al., 2006) and for the flaviviruses we recently demonstrated diverging C-terminal PBMs within the TBEVNS5 and WNVNS5 proteins, -SII and -TVL respectively (Werme et al., 2008). In addition, TBEVNS5 contain a novel internal PBM within the MTase domain that recognizes human Scribble (hScrib), regulating synaptic membrane exocytosis-2 (RIM2) and Zonula occludens-1 (ZO-1) (Ellencrona et al., 2009; Werme et al., 2008). The interaction between TBEVNS5 and hScrib was further found to be important for blockage of the innate interferon response in mammalian cells (Werme et al., 2008), and in blockage of NGF induced neurite outgrowth in PC12 cells (Wigerius et al., 2010).

Immense variations have been observed in the PBMs of different flavivirus NS5 proteins. However, the biological relevance of PDZ binding especially regarding replication, which is one of the best-characterized functions of the NS5 protein, remained largely elusive. Virus replicons provide a useful tool for studies of fundamental viral processes, such as replication and cellular tropism (Khromykh et al., 2001; Varnavski et al., 2000; Yamshchikov et al., 2001). Here we have used the genome of a Swedish TBEV (strain Torö-2003) as template to develop a DNA based sub-genomic replicon expressing the firefly luciferase gene (luc). By introducing suitable mutations into the replicon we show that the PBMs in TBEVNS5 influences virus replication. Furthermore, investigation of the C-terminal PBMs of TBEVNS5 and WNVNS5, revealed new host-binding partners. Taken together, our results highlight PDZ domains as an important target during flavivirus host invasion that could potentially serve as a target for novel antiviral therapies.

2. Materials and methods

2.1. Construction of DNA based TBEV sub-genomic replicons

A cloned TBEV strain (Torö-2003) (AH013799) (Melik et al., 2007), was used as the template to construct TBEV replicons expressing luciferase as a reporter. This replicon was identical to the replicon described in (Wigerius et al., 2010) (Fig. 1A), except that the EGFP gene was replaced with luc. Briefly, the replicon is driven by the CMV promoter expressing the 5′-untranslated region (UTR) and the 3′-UTR flanking an open reading frame including 60 nucleotides of the C gene fused in frame with the luciferase gene, the autoprotease gene of Foot and mouth disease virus 2a (FMDV 2a), 84 nucleotides of the E gene and all the NS proteins. The hepatitis delta virus antigenomic ribozyme (HDVr) sequence was inserted immediately downstream of the TBEV 3′-end followed by the Simian virus 40 (SV40) polyadenylation signal (pA) (Fig. 1A). To generate the control replicon, TBEVNS5Stop, two stop codons were introduced within the NS5 protein replacing residues G^9 and W^{12}, respectively (Fig. 1A). In addition, replicons expressing NS5 with

simultaneous mutations in either one or both PBMs ($YS^{223} \rightarrow AA$ and $SII^{903} \rightarrow AIA$) (Fig. 1B) were generated by directed mutagenesis.

2.2. Plasmids

All recombinant DNA techniques and cloning procedures were carried out by standard procedures (Sambrook et al., 1989). The TBEVNS5 and TBEVNS3 (Torö-2003, AH013799) and WNVNS5 (M12294) genes were amplified by PCR introducing suitable endonuclease restriction sites. Full-length NS5, NS5 mutants and NS3 were cloned into the pPiczB plasmid for yeast expression and pEYFP-C1 (Clontech) or pKH3 (kindly provided by Dr Ian Macara and Ben Margolis) for mammalian cell expression. The QuikChange XL-Site-Directed mutagenesis kit (Stratagene) was used to introduce amino acid changes following the manufacturer's instructions. To verify introduced mutations the constructs were sequenced at Eurofins MWG Operon, Ebersbeg, Germany.

2.3. Yeast protein expression and crude extract preparation

The pPiczB-NS5 plasmids were electroporated into *Pichia pastoris* and introduced into the genome according to the manufacturer's instructions (Invitrogen). *P. pastoris* was grown at 30 °C for 72 h supplemented with 0.5% methanol every 24 h to induce high NS5 expression. The Cells were lyzed with acid-washed glass beads (Sigma) according to the manufacturer's instructions and sonicated in 50 mM sodium phosphate buffer, pH 7.4, 5% glycerol and 1 mM phenylmethylsulfonyl fluoride (PMSF), and the supernatant was recovered after 10 min centrifugation at 12,000 × g, 4 °C.

2.4. Luciferase assay

COS-7 cells (1×10^5) were seeded into 12 well plates and were transfected with the different TBEV replicons (TBEVrep, TBEVrepYS → AA, TBEVrepSII → AIA, TBEVrepYS/SII → AA/AIA and TBEVNS5Stop) after 12 h, respectively. 350 ng of replicon plasmids was mixed with 100 ng pGL4.74[hRluc/TK]–a reporter constitutively expressing Renilla luciferase (Promega) and were transfected with lipofectamin 2000 according to manufacturer's instructions (Invitrogen). Cells were lyzed every 12 h post-transfection for 72 h using the Dual-Luciferase™ Reporter Assay according to manufacturer's instructions (Promega). Measurements for lucifearase were assayed in Lumi-star luminometer in triplicate in three independent experiments and the ratios of firefly luciferase to renilla luciferase were calculated and presented as relative luciferase units (RLU). The rmcdr package in the software application R was employed to analyze data. Statistical differences between means were determined using general linearized model followed by Tukey's post hoc test. Values are presented as mean ± SD. Experimental reproduction of results and the amount of data in account of statistical evaluations is indicated in figure legends.

2.5. siRNA treatment

Targeting and non-targeting siRNAs were designed by ambion. The hScrib-targeting double stranded siR-NAs was previously reported (Takizawa et al., 2006), and were #1: 5′-CAGGATGAAGTCATTGGAACA-3′ and #2: 5′-CCGCAGGAGGAGGATGGAGAA-3′. For the transfection of siRNAs, each siRNA (Final concentration 10 nM) was mixed with 350 ng of replicon plasmids and 100 ng pGL4.74[hRluc/TK], and transfected and assayed as above.
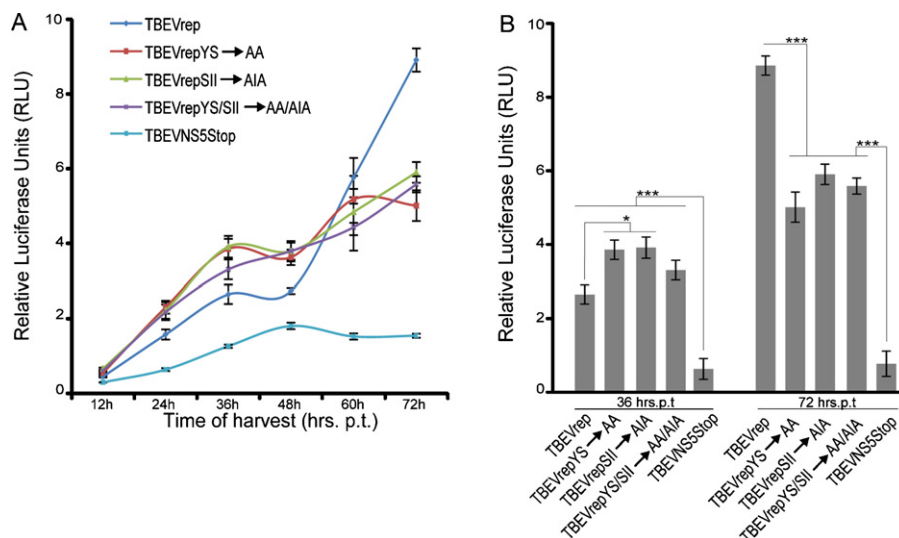
Fig. 2. Mutations in the PBMs of NS5 constrains TBEVrep activity. (A) COS-7 cells (10^5) were co-transfected with plasmid pGL4.74[hRluc/TK] (100ng) and replicons plasmids TBEVrep, TBEVrepYS → AA, TBEVrepSII → AIA, TBEVrepYS/SII → AA/AIA (350 ng respectively) and were lyzed for subsequent analysis with the dual luciferase assay (Promega) at the indicated time-points post-transfection (hrs. p.t.). Diagrams show the ratio of firefly luciferase to renilla luciferase expression with mean ± SD from three independent experiments. (B) Statistical data analysis of TBEVrep activity at 36 and 72 h, respectively. Data show RLU and error bars represent mean ± SD from three independent experiments in triplicate ($n = 9$). Asterisks indicate significant differences between replicon activities, $^*p < 0.05$ and $^{***}p < 0.0005$.

2.6. Antibodies and reagents

A rabbit polyclonal anti-NS5 antibody was raised against the NS5 peptide NH_2-CRFLEFEALGFLNEDHW^{498}-CONH$_2$ conjugated with the carrier protein, Keyhole Limpet Hemocyanin. Initial injection with 200 µg of protein emulsified in Freund's incomplete adjuvant was followed by 100 µg of protein over a period of 12 weeks. Antiserum was verified by ELISA and immunoblotting tests after 14 weeks. Affinity purification was performed using UltralinkTM Iodoacetyl and the antibodies were eluted in a nondenaturing elution medium at pH 7.0 followed by buffer exchange into PBS pH 7.4 (Agrisera). Goat anti-Scrib antibody (C-20): sc-11049, Goat anti-Scrib antibody (K-21): sc-11048, mouse anti-HA antibody (12CA5): sc-57592, rabbit anti-GIPC (H-55): sc-25556, rabbit anti-ZO-2 (H-110): sc-11448 and mouse anti-GFP (B-2): sc-9996 were from Santa Cruz, Biotechnology. Donkey anti-mouse Alexa488 (A21202) and goat anti-rabbit Alexa594 invitorgen (A11037) were from Invitrogen. Rabbit polyclonal anti-αTubulin antibody (#2144) was from Cell Signaling and protein G-sepharose beads was from Millipore. Protease inhibitor cocktail was from SIGMA.

2.7. PDZ array

PDZ array membranes were washed with 1× wash buffer and incubated with 1× blocking buffer according to the manufacturer's instructions (Panomics). The membranes were incubated with equal amount of WNVNS5 and TBEVNS5 crude extracts (1:1 in 1× resuspension buffer), overnight at 4°C. PDZ interactions were detected with the anti-NS5 primary antibody and anti-rabbit-HRP secondary antibody visualized using a chemoluminescence imagining system. Positive protein interactions were visualized as dark spots using a chemoluminescence imaging system. As a negative control, the membranes were treated similarly with crude yeast extract. Rows F and columns 9, 18 and 27 show positive protein controls.

2.8. Cell culture

The fibroblast like-cells, COS-7 is a cell-line derived from kidney of the African green monkey and is well known for expressing heterologous proteins at high levels. The epithelial dog madindarby canine kidney (MDCK) cell line has a cell morphology making it suitable for co-localization studies at the cellular periphery. COS-7 and MDCK cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen), containing 10% fetal bovine serum (FBS) and grown at 37°C in the presences of 5% CO_2.

2.9. Co-immunoprecipitation and immunoblots

For co-precipitation of endogenous ZO-2 and GIPC, COS-7 cells were grown in 6 well plates and were transfected with plasmid expressing HA-TBEVNS3, HA-TBEVNS5, HA-TBEVNS5YS → AA, HA-TBEVNS5SII → AIA and HA-TBEVNS5YS/SII → AA/AIA (2 µg respectively). 24 h post-transfection, the cells were lyzed in 200 µl/well radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris–HCl, pH.7.5, 135 mM NaCl, 1% Triton-X 100, 0.5% sodiumdeoxcholate, 5 mM $MgCl_2$ 1 mM EDTA, 1 mM Na_2VO_4 and protease inhibitor cocktail) for 30 min at 4°C. Cleared lysates were incubated with either 2 µg anti-ZO-2 or anti-GIPC (or anti-HA for reciprocal co precipitation) antibody overnight at 4°C, followed by incubation with protein G-sepharose beads for 5 h. The beads were collected by centrifugation 2000 × g for 1 min and washed 4 times in ice-cold RIPA buffer. Bound proteins were eluted with 2× laemmli sample buffer and subjected to SDS-PAGE and immunoblot analysis. Protein were separated in 9–12% SDS-PAGE gels and transferred to nitrocellulose membranes for detection with anti-HA (1:500), anti-α-Tubulin (1:1000), anti-GIPC (1:200), anti-ZO-2 (1:200) or anti-Scrib (1:200) antibodies.

2.10. Immunofluorescence

The pEYFP-C1-NS5 constructs were transiently transfected into MDCK cells, which were fixed for 10 min at −20°C with methanol/acetone (1:1) at 24 h post-transfection. The cells were incubated with primary, mouse anti-GFP (1:500) and either rabbit anti-ZO-2 (1:500) or rabbit anti-GIPC (1:500) antibodies overnight followed by 1 h incubation with goat anti-rabbit Alexa594 (1:1000) and donkey anti-mouse Alexa488 (1:1000). Fluorescence was visualized using confocal laser scanning microscopy (CLSM).
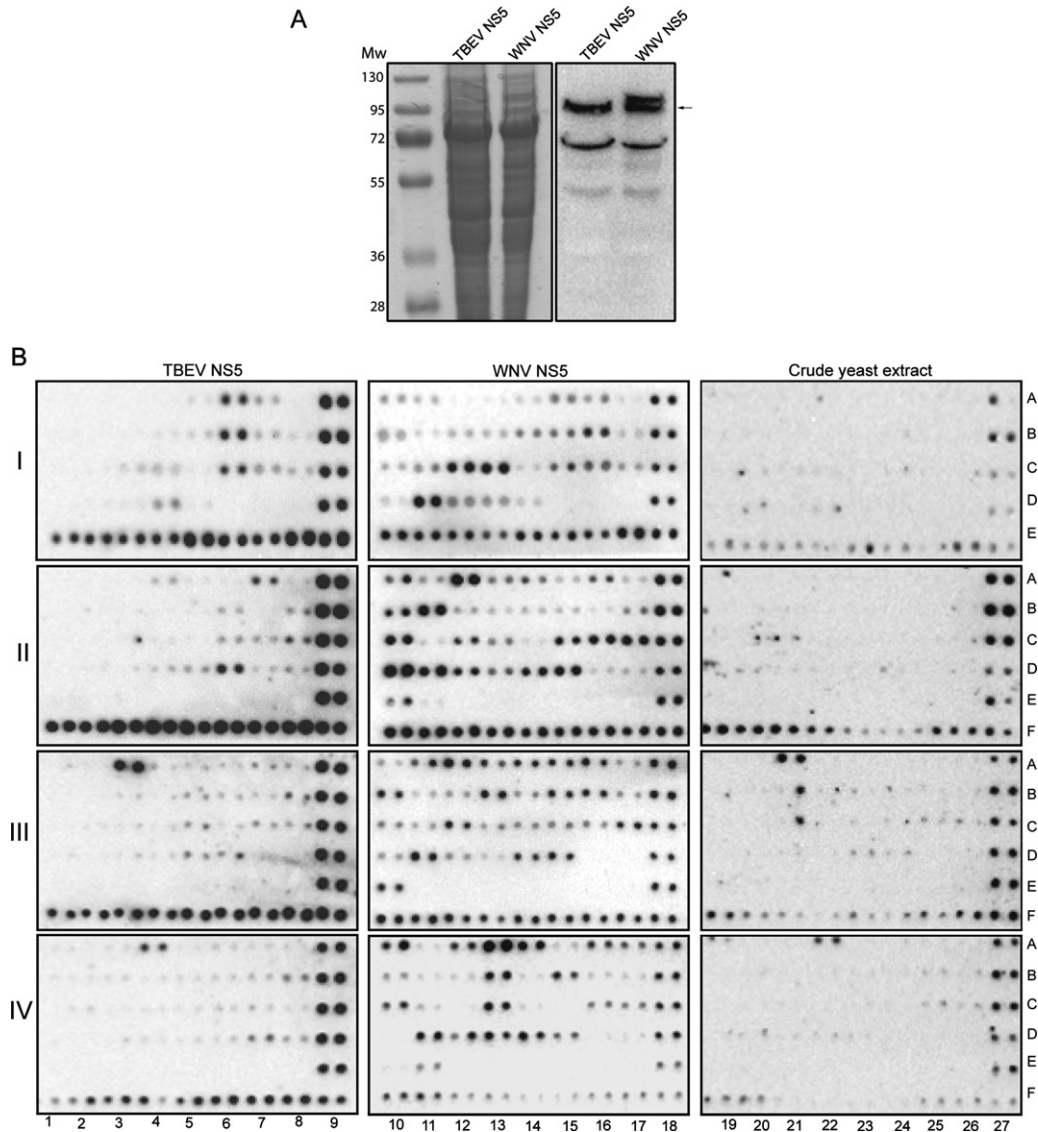
Fig. 3. TBEVNS5 and WNVNS5 proteins interacts with domains in the PDZ protein binding array (A) TBEVNS5 and WNVNS5 proteins expressed in *P. pastoris* were resolved on SDS-PAGE and visualized by Coomassie Brilliant Blue staining (left-panel), or developed by immunoblotting with anti-NS5 antibody (right-panel). The expected size of NS5 (103 kDa) is indicated with an arrow. (B) Extracts of *P. pastoris* with expressed TBEVNS5, WNVNS5 or crude yeast extract (control), were assayed respectively for their ability to bind 123 human PDZ domains on four membrane filters detected with NS5 specific antibody (I to IV; from Panomics), black dots indicate interaction.

3. Results

3.1. TBEV replication depends on the PBMs within NS5

We have previously highlighted the presence and function of both C-terminal and internal PBMs within the TBEV replication protein, NS5 (Ellencrona et al., 2009; Werme et al., 2008; Wigerius et al., 2010). As NS5 contains several essential enzymatic activities required for virus replication we postulated that these motifs could have direct or indirect roles in this process. To study this, an established DNA based TBEV replicon model was used (Wigerius et al., 2010), where the eGFP reporter gene was replaced with *luc*, which generated a luciferase expressing replicon (TBEVrep) (Fig. 1A). We also constructed a control replicon that lacked the NS5 protein (TBEVNS5Stop) (Fig. 1A). By mutating the PBMs of NS5, YS[263] and SII[903] (Fig. 1B), individually and in concert, we generated three additional replicons, TBEVrepYS → AA, TBEVrepSII → AIA and TBEVrepYS/SII → AA/AIA.

The replicons were transiently transfected into COS-7 cells and their replication rates were monitored in a luciferase time line assay over 72 h post-transfection (Fig. 2A). TBEVrep generated a clear biphasic pattern with peeks at 36 h and 72 h, respectively. The data includes luciferase expressed from the CMV promoter, but the level of expression was significantly higher than TBEVNS5Stop, which suggests that the RC of the TBEV underlay major parts of the activities recorded even at the earlier time point. The expression of TBEVrepYS → AA and TBEVrepSII → AIA were at moderately higher levels than the wild-type replicon at 36 h (Fig. 2B), which suggests that association with PDZ proteins might inhibit the initiation of TBEV replication. As expected, the expression of the TBEVrep reporter peaked at 72 h (Fig. 2). Interestingly, *luc* expression within TBEVrepYS → AA, TBEVrepSII → AIA and TBEVrepYS/SII → AA/AIA transfected cells were significantly reduced at 72 h (Fig. 2B), indicating that host associations with both PBMs enhances later TBEV replication, which could be linked to e.g. reduced capacity of the virus to evade immune responses of the host.

The internal PBM within NS5 is important for the association with hScrib (Werme et al., 2008), and to investigate if this association affects flavivirus replication we repeated the experiment in hScrib depleted cells. The activities of the replicons were

Table 1
Domains detected in the NS5 binding PDZ arrays.

TBEVNS5	Position	PDZ domain	Full name
Array I	A6	CSKP/CASK	Calcium/calmodulin-dependent serine protein kinase
	B6	GIPC	GIAP C-terminus interacting protein
	C6	ZO-2-domain 1	Zonula occludens protein 2
Array II	A7	KIAA1719-domain 4	Glutamate receptor interacting protein 2
	D6	IL16(2)-domain 3	Interleukin 16 isoform 2, lymphocyte chemoattractant factor

WNVNS5	Position	PDZ domain	Full name
Array I	B16	HtrA2/Omi	High temperature requirement protein
	C12	OMP25	Mitochondrial outer membrane protein 25
	C13	hCLIM1	Human 36 kDa carboxyl terminal LIM domain protein
	C15	ZO-2-domain 1	Zonula occludens protein 2
	C16	hPTP1E-domain 1	Human tyrosine-protein phosphatase non-receptor type 13
	D11	RIL	Reversion-induced LIM protein
Array II	A10	KIAA0300-domain 6	PDZ domain-containing protein 2
	A12	KIAA0316	FERM and PDZ domain-containing protein 4
	A16	KIAA1719-domain 4	Glutamate receptor interacting protein 2
	B10	KIAA1526-domain 3	Whirlin
	B11	MAST205	Microtubule associated serine/threonine kinase 2
	C10	PALS1-domain 3	Pals1-associated tight junction protein
	C12	FLJ23209-domain 1	PDZ domain containing 7
	C15	FLJ00011	FLJ00011 protein (fragment)
	C16	E3KARP-domain 1	Solute carrier family 9
	C17	E3KARP-domain 2	Solute carrier family 9
	D10	NHERF1-domain 1	Solute carrier family 9
	D11	ZO1-domain 1	Tight junction protein 1 (Zonula occludens)
	D12	ZO1-domain 2	Tight junction protein 1 (Zonula occludens)
	D14	SDCBP-domain 2	Syndecan binding protein (syntenin)
	D15	IL16(2)-domain 3	Interleukin 16 isoform 2, lymphocyte chemoattractant factor
Array III	A11	MAG13-domain 4	Membrane associated guanylate kinase-related
	A13	MAG13-domain 6	Membrane associated guanylate kinase-related
	A14	BAI1-domain 2	Brain-specific angiogenesis inhibitor associated protein 1
	A15	BAI1-domain 3	Brain-specific angiogenesis inhibitor associated protein 1
	A16	BAI1-domain 4	Brain-specific angiogenesis inhibitor associated protein 1
	B10	BAI1-domain 6	Brain-specific angiogenesis inhibitor associated protein 1
	B13	AIP1-domain 4	Atrophin-1 interacting protein 1
	B15	AIP1-domain 6	Atrophin-1 interacting protein 1
	B16	hPTP1E-domain 3	Human tyrosine-protein phosphatase non-receptor type 13
	C12	GRIP1-domain 4	Glutamate receptor-interacting protein 1
	C17	SCRIB1-domain 4	Scribble
	D11	PARD3-domain 3	Partitioning-defective protein 3 homolog; atypical PKC
	D14	TIP1	Tax interacting protein 1
	D15	SDB2-domain 2	Syntenin-2beta
	E10	PDZ-Pos	PDZ Domain positive control for Kv1.4 ligand
Array IV	A10	MUPP1-domain 6	Multiple PDZ domain protein
	A12	MUPP1-domain 2	Multiple PDZ domain protein
	A14	MUPP1-domain 13	Multiple PDZ domain protein
	A16	DLG3-domain 2	Synapse-associated protein 102
	B13	DLG5-domain 2	Discs, large homolog 5
	B15	PAR6B	Partitioning defective-6 homolog beta
	C13	LIN7C	Lin7 homolog C
	D11	PDZK1-domain 2	PDZ domain containing protein 1
	D12	SNTB1	Beta-1-syntrophin
	D13	SNA1	Acidicalpha 1 synthropin; dystrophin-associated protein A1
	D14	SHANK1	SH3 and multiple ankyrin repeat domains 1

overall reduced (data not shown). However, as the activity of the TBEVNS5Stop also was significantly reduced we believe that the reduction was a general effect on the host cell viability and the transcription and translation machinery. Otherwise, the pattern between the different replicons was similar as in untreated cells, indicating that the NS5-hScrib association has no or limited direct effect on TBEV replication (data not shown).

3.2. TBEVNS5 and WNVNS5 associate with different PDZ domains

No significant difference in luciferase expression was observed between the TBEV replicons with either single or double PBM mutations (Fig. 2), which suggest that the replication might depend on a single protein complex associating simultaneously at both

sites. In addition the C-terminal sequence might have additional roles in PDZ dependent host-targeting of flaviviral NS5. As sequence analysis of TBEVNS5 and WNVNS5 has revealed C-terminal PBMs -SII and -TVL, respectively (Werme et al., 2008), we expressed both proteins in *Pichia pastoris*. The genes were cloned into the pPiczB vector with their intact stop codons to express untagged native proteins. For detection, we developed a polyclonal peptide antibody against a sequence within the RdRp domain, which recognizes both the TBEV and the WNV proteins. The NS5 constructs were introduced into the yeast genome and proteins were expressed by methanol induction for 3 days as outlined in methods. To produce crude extracts containing TBEVNS5 and WNVNS5, the cells were lyzed, centrifuged and the cytoplasmic fractions were separated on SDS-PAGE and analyzed by immunoblotting (Fig. 3A). The NS5 specific antibody revealed a band at the expected size of TBEVNS5
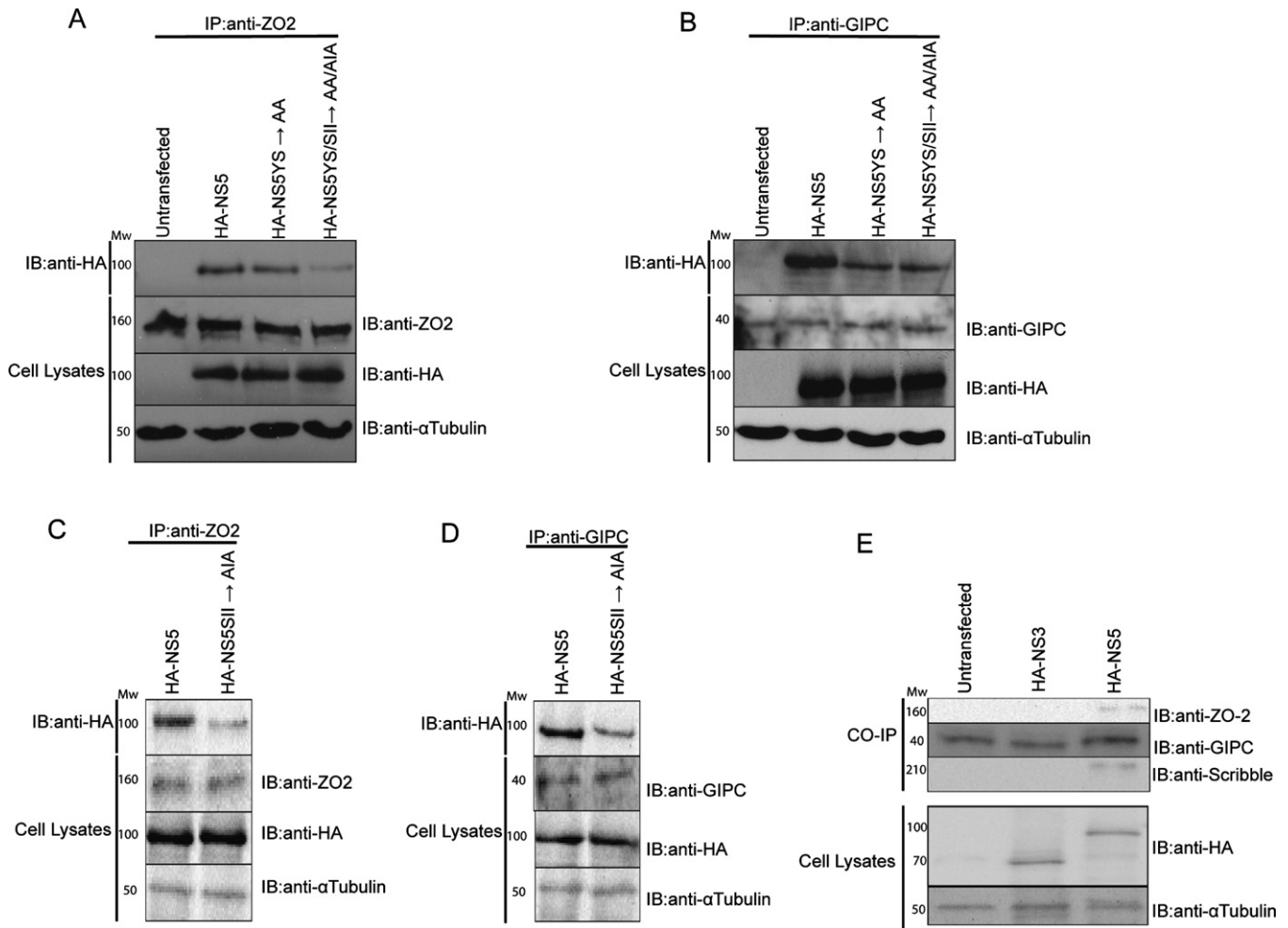
Fig. 4. TBEVNS5 interacts with ZO-2 and GIPC in cell culture. (A) COS-7 cells were transiently transfected with 2 μg each of plasmid expressing TBEV HA-NS5, HA-NS5YS → AA or HA-NS5YS/SII → AA/AIA. Cell lysates were prepared 24 h post-transfection and analyzed by SDS-PAGE with anti-ZO-2 and anti-HA antibodies or was immunoprecipitated (IP) with anti-ZO-2 overnight. Precipitated proteins were resolved by SDS-PAGE and was immunoblotted (IB) developed with anti-HA antibody. (B) COS-7 cells were transiently transfected with 2 μg each of plasmid expressing TBEV HA-NS5, HA-NS5YS → AA or HA-NS5YS/SII → AA/AIA. Cell lysates were prepared 24 h post-transfection and was analyzed by SDS-PAGE with anti-GIPC and anti-HA antibodies or was IP with anti-GIPC overnight. Precipitated proteins were resolved by SDS-PAGE and IB with anti-HA antibody. (C) COS-7 cells were transiently transfected with 2 μg of plasmid expressing TBEV HA-NS5SII → AIA and cell lysates were prepared and analyzed as in A. (D) COS-7 cells were transiently transfected with 2 μg each of plasmid expressing TBEV HA-NS5SII → AIA and cell lysates were prepared and analyzed as in B. (E) The reciprocal control was made in COS-7 cells transiently transfected with 2 μg of HA-NS3 or HA-NS5 plasmids, respectively. Cell lysates were prepared 24 h post-transfection and was analyzed by SDS-PAGE with anti-HA and anti-α-Tubulin antibodies or IP with anti-HA overnight. Precipitated proteins were resolved by SDS-PAGE and IB developed with anti-ZO-2, anti GIPC or anti-Scrib antibodies, respectively. Note, faint overlapping bands detected at the same size as GIPC (40 kDa) are unspecific.

(103 kDa), but also an additional band around 70 kDa (Fig. 3A). The 70 kDa band was also present in non-induced yeast extracts, which indicates unspecific NS5 antibody cross-reactivity (data not shown). The WNVNS5 extract generated similar results but interestingly a double band of NS5 appeared (Fig. 3A). This indicates that there was a difference in the modification status of the two proteins, possibly due to phosphorylation or ubiquitination events, which previously has been reported for flaviviral NS5 proteins (Kapoor et al., 1995; Taylor and Best, 2011).

To investigate the binding capacities for TBEVNS5 and WNVNS5 the extracts were panned in the TranSignal PDZ Domain arrays (Panomics). Both TBEVNS5 and WNVNS5 were able to bind PDZ domains, and based on the comparison with a previous study of the internal PBM (Ellencrona et al., 2009), the detected TBEV interactions seems to involve recognition mainly via the C-terminal motif. Large differences were observed in the binding specificity for NS5 between the two virus species (Fig. 3B) (Table 1). The C-terminal-SII motif of TBEVNS5 seems to be more specific compared to the promiscuous -TVL motif of WNVNS5 (Fig. 3B) (Table 1), however further studies and mutational analysis are required to verify these

interactions. The presence of serine at the −2 position within the TBEVNS5 motif might be a main factor explaining the observed difference, as substitution of S–T at this position results in a twofold increase in PDZ domain affinity (Kurakin et al., 2007). Two of the detected interactions (Fig. 3B, array III, A3 and A12), (Fig. 3B, array IV, A4 and A13) are not specific for the NS5 proteins as they also was observed by the non-induced control extracts (Fig. 3B, array III, A21; array IV, A22).

TBEV is neuroinvasive, but despite our understanding of the clinical outcome, the involvement of factors and a mechanism behind severe TBE is presently unclear. TBEVNS5 was found to associate with five PDZ domain proteins, ZO-2 PDZ domain 1 (Fig. 3B, array 1, C6), GIPC (Fig. 3B, array 1, B6), CASK (Fig. 3B, array 1, A6), GRIP2 PDZ domain 4 (Fig. 3B, array 2 A7) and IL-16 PDZ domain 3 (Fig. 3B, array 2, D6) and interestingly all these proteins have key roles in neuronal cells (Choi and Rothman, 1990; Hsueh, 2006; Kurschner and Yuzaki, 1999; Kuruganti et al., 2002; Lee et al., 2009; Osten et al., 2000; Yi et al., 2007). The only protein that associates strongly with both the NS5 proteins was IL-16 (Fig. 3B, array 2, D6 and D15) (Table 1).

3.3. TBEVNS5 associate in vivo with endogenous ZO-2 and GIPC

To investigate whether the PDZ motifs of NS5 were important for binding ZO-2 or GIPC in a cellular context, TBEV HA-NS5 and mutated proteins HA-NS5YS → AA and HA-NS5YS/SII → AA/AIA were expressed in COS-7 cells. 24 h post-transfection the cells were lyzed and the protein extract were immunoprecipitated with anti ZO-2 or GIPC antibodies, respectively. We found that endogenous ZO-2 co-precipitated with NS5 and with NS5YS → AA. However, the binding capacity of NS5YS/SII → AA/AIA with ZO-2 was impaired (Fig. 4A). These results support that TBEVNS5 associates with ZO-2 in mammalian cells mainly via its C-terminal PDZ motif. Surprisingly, the TBEVNS5YS → AA demonstrated reduced capacity to bind GIPC suggesting that internal PDZ recognition also is important for binding GIPC (Fig. 4B). To clarify the role of the C-terminal PBM we generated and expressed HA-NS5SII → AIA, which demonstrated reduced capacity to bind both ZO-2 and GIPC in vivo (Fig. 4C and D). We further addressed the significance of these interactions by reciprocal precipitations with expressed HA-NS5, including the control proteins TBEV HA-NS3 and hScrib. The cells were lyzed 24 h post-transfection and the protein extract were immunoprecipitated with anti-HA antibody and immunoblotted with specific antibodies for ZO-2, GIPC and hScrib, respectively (Fig. 4E). The results further support that ZO-2, GIPC and hScrib specifically co-precipitate with TBEVNS5 in cell culture (Fig. 4E).

3.4. Co-localization of TBEVNS5 with ZO-2 and GIPC

As TBEVNS5 binds to ZO-2 and GIPC we wanted to further examine the cellular localization of the putative NS5-ZO-2 and NS5-GIPC complexes. A construct containing the TBEVNS5 gene cloned into pEYFP-C1 (Clontech) (Ellencrona et al., 2009) was transiently transfected into MDCK cells and were analyzed by CLSM after 24 h. TBEVNS5 was detected in the cytoplasm, the nucleus and at the cellular periphery (Fig. 5, upper panels). As expected, ZO-2 and GIPC stained primarily at the plasmamembrane (Fig. 5, upper-middle panels), and co-localized with TBEVNS5 at the cell cortex of cells with clear adherence to neighboring cells (Fig. 2, lower panels).

4. Discussion

To our knowledge our observations are the first to report on PDZ protein binding connected to flavivirus replication. Here we show that host protein interactions involving PBMs of TBEVNS5 could have a direct role in virus replication. As no additive effect was seen comparing TBEVrepYS/SII → AA/AIA with TBEVrepYS → AA and TBEVrepSII → AIA, we believe that the binding could involve a single complex of host PDZ proteins. Nevertheless, as mutations at the PBMs may affect structures compromising MTase and RdRP we cannot currently rule out that the observed results also include enzymatic impairment of NS5.

As the hScrib "knock-down" had no clear effect on TBEV replication, further studies on the replicons, e.g. in ZO-2 and GIPC depleted cells, are required to clarify specific host factors involved in TBEV replication.

Recent reports of human and avian IAV highlight differences within the C-terminus of NS1 as vital for PDZ protein binding influencing viral replication. For example, in mice, a recombinant IAV expressing NS1 with a PBM changed into an avian specific sequence, significantly increased the virulence of the virus (Jackson et al., 2008). Moreover, it was recently reported that species-specific differences within IAV NS1 are connected to host adaptation, replication and virulence (Soubies et al., 2010). Introducing the C-terminal NS1 sequence RSKV (typically found in
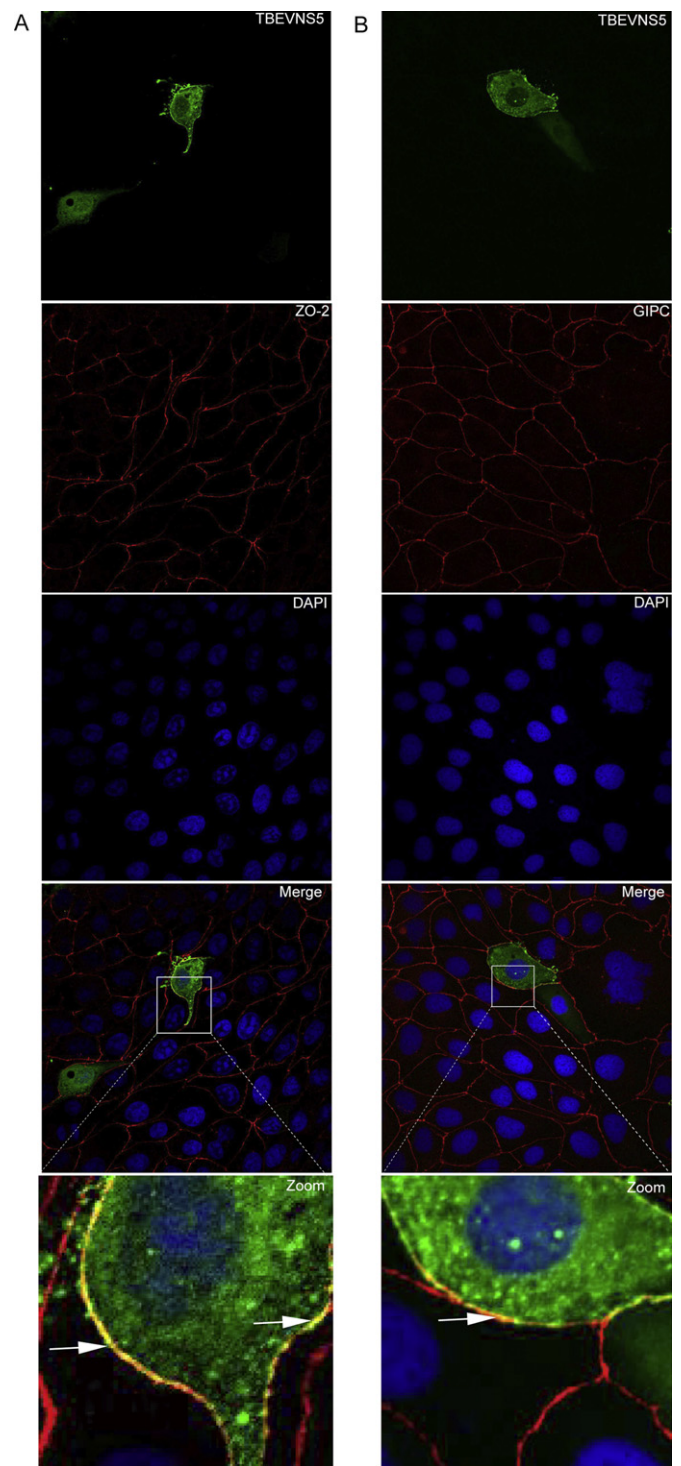

Fig. 5. Co-localization of TBEVNS5 with endogenous proteins ZO-2 and GIPC. CLSM images of MDCK cell expressing transfected YFPNS5 and endogenous ZO-2 and GIPC, respectively. Cells were fixed and stained with anti-ZO-2 or anti-GIPC antibody, respectively, followed by alexa488- and alexa594-conjugated secondary antibodies. Nuclear DAPI staining is shown in the middle panels. The lower panels show the merge, which revealed co-localization (indicated by arrows) of TBEVNS5 and the endogenous proteins at the cellular periphery, 24 h post-transfection.

human IAV) increased replication rate of avian IAV, H7N1 (low pathogenic) in human cells and duck whereas introduction of the ESEV sequence (typical for avian IAV) increased IAV replication rate and pathogenicity in mice (Soubies et al., 2010). In this perspective, as flaviviruses also utilize a broad host repertoire replicating in both

vertebrate and invertebrate cells, it would be interesting to further study putative effects of PDZ associations on TBEV replication in the Tick host.

GIPC and ZO-2 interacts with TBEVNS5 under physiological conditions and it appears that the C-terminal TBEVNS5 binding site is the more vital binding motif for the ZO-2 association. ZO-2 belongs to the MAGUK family (Membrane-associated guanylate kinases) and contains 3 PDZ domains. The protein is highly homologues to ZO-1 and shuttle between the nucleus and the tight junction (TJ) in epithelial cells (Islas et al., 2002; Itoh et al., 1997). ZO-2 has also been found to interact and down-regulate activator protein-1 (AP-1) dependent expression (Betanzos et al., 2004). For some RNA viruses, e.g. rotavirus, activation of the AP-1 plays an important role for optimal replication (Holloway and Coulson, 2006). Possibly there are functional roles for TBEVNS5-ZO-2 binding connected to AP-1 activity, which will be further investigated in a future study.

It has been shown that the PDZ domain 3 and 4 of human Scribble interacts with ZO-2 while mutations in the LLR delocalize Scribble from the TJ and impair the association (Metais et al., 2005). As we previously showed that an internal motif in TBEVNS5 binds specifically to Scribble–PDZ4 (Werme et al., 2008), our new data indicate that TBEVNS5 could be targeting the Scribble–ZO-2 complex using both PBMs.

GIPC, contain on PDZ domain, and is detectable in clathrin-coated pits and found throughout the neuronal cell at both presynaptic and postsynaptic locations (Miaczynska et al., 2004). Moreover, GIPC plays a role in trafficking of membrane proteins including the neurotrophic tyrosine kinase receptor type 1 (TrkA), and has been suggested to provide a link between TrkA and G protein signaling pathways (Jeanneteau et al., 2004; Lou et al., 2001; Varsano et al., 2006; Yano et al., 2006). GIPC also binds the NR2 subunit of the NMDA (Yi et al., 2007). GIPC has effects on cellular signaling pathways, which indicate that this TBEVNS5 targeting could impede important functions within the host cell during infection.

Based on our PDZ binding array, we observe that WNVNS5 might contain a more promiscuous binding motif in the C-terminus compared to TBEVNS5 (Table 1). The only PDZ domain which seems to be targeted strongly by both proteins are IL-16, a cytokine with chemotactic properties (Glass et al., 2006). IL-16 stimulation of peripheral blood mononuclear cell (PBMC) results in secretion of other interleukins and tumor necrosis factor-α (TNF-α), suggesting that IL-16 might be important in initiating and/or sustaining inflammatory response (Mathy et al., 2000). In the brain, IL-16 occurs as an intracellular neuronal variant, NIL-16, which interacts with the NR2A subunit of the NMDA receptor at the cellular periphery (Kurschner and Yuzaki, 1999).

Altogether several of the revealed interactions, imply a neuronal PDZ complex associated with the NMDA receptor, which might be targeted by TBEVNS5. As TBEV is a neurotropic virus the observed interactions might be factors affecting disease outcome. One important future task will be to further characterize the functional significance of PDZ dependent flavivirus-host interactions within the viral life cycle in a neuronal context.

Acknowledgments

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