Effect of nicotinic acetylcholine receptor alpha 1 (nAChRα1) peptides on rabies virus infection in neuronal cells

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ABSTRACT

Rabies virus (RABV) is neurotropic and causes acute progressive encephalitis. Herein, we report the interaction of nAChRα1-subunit peptides with RABV and the effect of these peptides on RABV infection in cultured neuronal cells. Peptide sequences derived from torpedo, bovine, human and rats were synthesized and studied for their interactions with RABV using virus capture ELISA and peptide immunofluorescence. The results showed specific binding of the nAChRα1-subunit peptides to the RABV. In the virus adsorption assay, these peptides were found to inhibit the attachment of the RABV to the neuronal cells. The nAChRα1-subunit peptides inhibited the RABV infection and reduced viral gene expression in the cultured neuroblastoma (N2A) cells. Torpedo peptide sequence (T-32) had highest antiviral effect ($IC_{50} = 14 \pm 3.01$ μM) compared to the other peptides studied. The results of the study indicated that nAChRα1-subunit peptides may act as receptor decoy molecules and inhibit the binding of virus to the native host cell receptors and hence may reduce viral infection.

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1. Introduction

Rabies encephalitis remains one of the important public health problems in Asian and African countries (Huang et al., 2015; Kabeta et al., 2015). The burden of rabies is highest among all the neglected tropical diseases with 1.91 million disability adjusted life years and the projected annual loss of US$ 6 billion (WHO, 2013). In the rabies bites, only prompt post-exposure prophylaxis (PEP) can protect the patients and fatalities occur in almost 100% of cases following the onset of clinical disease (Bourhy et al., 2010). Presently no pragmatic treatment methods are available and induction of coma is considered only as a part of supportive care in case of clinical rabies (Wilde and Hemachudha, 2015). RABV infections can be prevented by interfering at any of the steps in the viral life cycle. Attachment to the receptors and entry of the virus into the host cells is the initial critical step in the virus life cycle. Rabies virus has receptors in the nervous system and the neuronal receptor peptides may act as potential antiviral molecules by blocking the attachment and entry of the RABV into the host cells.

RABV is a prototype neurotropic virus and belongs to the genus *Lyssavirus* in the *Rhabdoviridae* family (King et al., 2012). It has small negative sense single stranded RNA genome of 12 kb which codes for five different proteins, namely the nucleoprotein, the phosphoprotein, the matrix protein, the glycoprotein and the large RNA dependent RNA polymerase (Finke and Conzelmann, 2005). Rabies virus glycoprotein (RVG), organized as trimers is the only protein exposed on the virus particle. It interacts with the host cell, mediates pH dependent fusion and promotes viral entry from the peripheral site to the nervous system (Roche and Gaudin, 2004). Further RVG is the major contributor to the pathogenicity and is involved in trans-synaptic spread of RABV within the CNS (Pulmanausahakul et al., 2008; Klingen et al., 2008). It is less clear about the host molecule to which the RVG binds. In vivo, RABV was found to multiply in neurons, muscle fibers and salivary gland cells however the virus can be adapted and propagated in various continuous cell types in vitro (Seganti et al., 1990). RABV restricted cell tropism in vivo indicates the presence of unique host cell receptors. Nicotinic acetylcholine receptor (nAChR) was the first identified receptor for RABV (Lentz et al., 1982). Other potential host cell receptors include neuronal cell adhesion molecule (NCAM) (Lafon, 2005) and low-affinity nerve growth factor (p75^{NTR}) (Tuffereau et al., 1998). However, RABV was able to infect mice which were deficient in either NCAM or p75NTR similar to wild-type mice (Tuffereau et al., 2007). This indicates that although these molecules may have role in infection process, they are not essential for entry of RABV. Earlier the interaction of purified nAChR with the RABV was confirmed in different experiments (Bracci et al., 1988; Gustka et al., 1996). The toxin loop identified within the RVG has homologous sequence to other neurotoxins and is

responsible for binding to the nAChRs (Lentz, 1990). Conformational studies identified an internal tetra-peptide of RVG toxin loop (RVG-29mer) as an essential part of the binding site to nAChR (Rustici et al., 1993). Besides being present in the muscle membranes at synaptic junctions, nAChRs are present in the central and peripheral nervous system with similar acetyl choline binding properties (Albuquerque et al., 2009). nAChRs may be involved in the entry of RABV at post-synaptic junctions and also spread within the nervous system (Schnell et al., 2010). Harnessing the binding ability of RVG to the nAChR, recent studies have shown the successful use of RVG-29mer peptide to deliver therapeutic molecules to the brain (Son et al., 2011; Kim et al., 2013; Gao et al., 2014). Considering the wide spread presence of nAChRs in the nervous system and the binding of RVG to these receptors, nAChR peptides may act as receptor decoy molecules for RABV. The binding of receptor peptides may prevent the attachment and entry of the RABV into the host neuronal cells which is the essential step in viral infectivity cycle.

In the present study, nAChRα1-subunit peptide variant sequences from different species were synthesized and tested for their interactions with RABV. The interacting peptides were evaluated against RABV infection in cultured neuroblastoma cells.

2. Experiments

2.1. Virus propagation in N2A cells and titration

N2A cells were obtained from the National Centre for Cell Science, Pune, India and grown at 37 °C under 5% CO_2 in Dulbecco's modified minimum essential medium (DMEM, Hyclone), supplemented with 10% fetal bovine serum (Hyclone). The mouse-brain-adapted rabies challenge virus standard 18 (RABV-CVS18) strain was propagated in the N2A cells. After three passages, the virus titer in the culture supernatants was determined by fluorescent focus unit (FFU) assay following standard method prescribed by world organization for animal health (OIE) (Smith et al., 1977). The culture supernatant had an infectious potency of 3×10 FFU/ml. This stock of virus was made into aliquots and used for further experiments. For virus attachment studies, concentrated virus preparations were obtained by mixing the culture supernatants with polyethylene glycol (8%), NaCl (2.2%) and phenyl methyl-sulfonyl fluoride (0.01%). The resulting solution was incubated overnight at 4 °C with gentle stirring followed by centrifugation at $15,000 \times g$ for 90 min and finally resuspended in the tissue culture medium to use in further experiments.

2.2. Peptide synthesis

Solid phase methodology with Fmoc chemistry was used to synthesize sequences of native nAChRα1-subunit peptides derived from torpedo, bovine, human and rat. These peptide sequences characteristically fall between 174 aa to 203 aa region of α1 subunit of the receptor. Phage display derived α-bungarotoxin binding peptides discovered earlier were also included in the present study (Moshe et al., 1997). Briefly after 1 h swelling of rink amide MBHA resin (Nova Biochem), 20% piperidine treatment was done to remove the protecting Fmoc group. The first Fmoc-amino acid (5 equivalent to the loading capacity of the resin) was activated with equivalent amount of 1-(Bis (dimethylamino) methylene)-1 H-benzotrizoliumhexaflurophosphate (1-) 3 oxide (HBTU) and 1-hydroxybenzotriazol (HOBT) and made to react with the resin in the presence of di-isopropyl ethylamine (DIEA). Coupling was allowed for 2 h followed by end capping with acetic anhydride. Loading/coupling efficiency was monitored at each steps of synthesis using Kaiser test (Kaiser et al., 1970). Subsequent deprotection, coupling and end capping were repeated till the completion of synthesis. Peptides were deprotected and cleaved from resin beads using a treatment of trifluoroacetic acid/phenol/thioanisol/1dodecanethiol/water (82.5:5:5:2.5:5 v/v) mixture for 4 h and precipitated in chilled dry diethyl ether.

2.3. Purification and characterization of peptides

Precipitated peptides in crude forms were purified by reversed-phase chromatography (RP-HPLC) on a C-18 semi-preparative column (7×300 mm; 10 μ particle size) using UFLC pump system (Shimadzu, Tokyo, Japan) fitted with photo diode array (PDA) detector. The binary gradient of water/acetonitrile having 0.1% TFA (v/v) was used for purification of peptides. The flow rate was kept at 1 ml/min and the major peaks were collected. The collected elutions were dried in Speed Vac concentrator (Eppendorf, Germany) and resuspended in HPLC grade water. Peptides were further analyzed for purity on analytical C-18 column (4×150 mm; 5 μ particle size). The identity and purity of the final products were confirmed by Ettan™ MALDI–TOF (matrix-assisted laser-desorption ionization-time-of-flight) MS (Amersham Biosciences) (Supporting materials). The sequences of the synthesized peptides and their physico-chemical properties were determined by using ProtParam algorithm at ExPASy (the Expert Protein Analysis System) provided by the Swiss Institute of Bioinformatics (SIB) (Table 1).

2.4. Virus capture ELISA

Microtitre plates were coated with 100 μg/ml peptides for 24 h at 4 °C. Plates were blocked with 1% BSA in Tris buffered saline (TBS) for 1 h at room temperature, followed by 2 h at room temperature with 10 μg per well of β-propiolactone (BPL) inactivated RABV. After extensive washing, plates were probed with 1:1000 dilution of monoclonal mouse anti-rabies IgG (Rab sc-57994, Santa Cruz) for 1 h at 37 °C. Plates were washed with PBS and a 1:2000 dilutions of chicken anti-mouse HRP conjugate (sc-2954, Santa Cruz) was added to each well, incubated for 1 h at 37 °C. The wells were washed with PBS and developed by using TMB substrate (Amresco) at room temperature for 20 min. Plates were read at 450 nm after addition of stopping solution (1 M H_2SO_4). The binding was calculated by determining the change in virus binding compared to the wells coated with BSA alone.

2.5. Cytotoxicity test for peptides in N2A cells

Cytotoxicity test for nAChRα1-subunit peptides was performed using the MTT method (Mosmann, 1983). Briefly equal numbers of cells (2.5×10 were seeded in each well of 96 wells culture plates. 50 μl of different concentration of peptides (6.25, 12.5, 25, 50, 100 & 150 μM) diluted in DMEM were added to the cells. 20% DMSO and DMEM alone were included as the positive and negative control groups respectively. Plates were incubated for 24 h at 37 °C, under a humidified 5% CO_2 atmosphere. The medium was removed and 50 μl of MTT solution was added to each well and incubated for 4 h. 100 μl of DMSO was added to the wells and gently rocked to solubilize formazan. Absorbances were measured using spectrophotometer at 540 nm. The CC_{50} was calculated as the concentration of the peptides that reduced the absorbance of treated wells to 50% when compared to the control wells.

2.6. Virus inhibition assay

Virus inhibition assay was performed using NIH test protocol, modified rapid fluorescent focus inhibition test (RFFIT) (Smith et al., 1973). N2A cells were seeded in 24 well plates on the previous day to reach 80–90% confluence. The cells were inoculated with peptide pretreated (0 to 50 μM of peptide) and untreated RABV-CVS18 at an MOI of 0.1 for 1 h at 37 °C. Following the adsorption, inoculum was removed and cells were incubated in DMEM containing 2% fetal bovine serum (FBS). The supernatants were collected at after 48 h (hpi), and stored at −80 °C for viral RNA isolation. The infected cells monolayers were fixed with 80% acetone to detect RABV in infected cells by direct immunofluorescence test using FITC-labeled antibody against RABV nucleocapsid protein (BioRad). Cell nuclei were counterstained with Prolong gold anti-fade reagent with DAPI (Invitrogen). The viral foci were counted in 6 random

Table 1
Physical and chemical properties of peptides.

Peptides	Sequence	M·Wt g/mol	pI	GRAVY	$t_{1/2}$ h	Stability
Torpedo-32 (T-32)	SGEWVMKDYRGWKHWVYYTCCPDTPYLDITYH	4014	7.1	−0.787	2	−5.9 (S)
Bovine-32 (C-32)	SGEWV**IKES**RGWKHWV**FYA**CCP**S**TPYLDITYH	3859	8.2	−0.400	2	−5.9 (S)
Human-32 (H-32)	SGEWV**IKES**RGWKH**SVTYS**CCPDTPYLDITYH	3758	7.1	−0.672	2	20.9 (S)
Torpedo-20 (T-20)	KDYRGWKHWVYYTCCPDTPY	2581	8.8	−1.265	1.3	−6.2 (S)
Bovine-20 (C-20)	K**ES**RGWKHWV**FYA**CCP**S**TPY	2445	9.5	−0.775	1.3	94.3 (U)
Rat-14 (R-14)	**ES**RGWKHWV**FYA**C	1668	10.1	−0.857	1.3	103.0(U)
Lpep-13 (L-13)	MRYYESSLKSYPD	1637	9.3	−1.354	30	28.1 (U)
DDD-14 (D-14)	FRYYESSLEPWDDD	1820	3.8	−1.579	1.1	46.5 (U)

Where pI is the theoretical isoelectric point; GRAVY, grand average of hydropathicity, positive values are hydrophobic; $t_{1/2}$ is estimated half-life in mammalian reticulocytes, in vitro; U, unstable; S, stable; the bold letters indicate changes in the amino acids in the peptide sequence.

fields and mean number of foci in the peptide treated and control wells were recorded. The IC$_{50}$ was calculated as the concentration of peptide that reduced the number of fields with fluorescent foci to 50% when compared with the negative control with plain medium.

2.7. Virus attachment experiment

The effects of receptor peptides on the attachment of RABV to host neuronal cells were studied using virus attachment study. N2A cells were seeded in 96 wells culture plates to reach near confluent layer. An increasing concentration of peptides were mixed with PEG–NaCl concentrated RABV (MOI of 6) and incubated for 1 h at 37 °C and chilled. Virus-peptide solutions were plated on N2A cells monolayer at 4 °C for 2 h with gentle rocking at every 15 min. The content removed, washed with cold PBS and fixed with 4% paraformaldehyde at room temperature followed by washing with PBS. Plates were blocked with 3% BSA in TBS for 1 h at room temperature and again washed with PBS. Primary mouse anti-rabies IgG (Rab sc-57994, Santa Cruz) was added (1:1000) and incubated for 1 h at 37 °C. Plates were washed with PBS and then probed with goat anti-mouse IgG-HRP (sc-2954, Santa Cruz) for 1 h at 37 °C. The plates were washed with PBS and developed using 100 μl of TMB at room temperature for 15 min. Plates were read at 450 nm after addition of 1 M of H_2SO_4 stop solution.

2.8. Real time PCR for viral gene expression

The RABV-L gene copy numbers were quantified in the culture supernatants using one-step quantitative real-time PCR. Known copies of the viral RNA were 10-fold serially diluted to generate a standard curve. The viral RNA was extracted using the QIAmp viral RNA mini kit (QIAGEN, Germany) and the qRT-PCR was performed using a SYBR Green Master Kit (QIAGEN, Germany). Triplicate reactions were performed for each sample, and a no template control (NTC) was included as a negative control. Absolute quantification was performed using an ABI7500 machine (Applied Biosystems, Foster City, CA). The results were analyzed using Sequence Detection Software Version 1.3 (Applied Biosystems, Foster City, CA).

2.9. Statistical analysis

Statistical analysis was performed using SPSS-20 software package. Data were compared between groups using one-way analysis of variance followed by post hoc test. Data from triplicates of two independent experiments were considered for analysis and presented as mean ± SEM. Differences up to $p < 0.01$ were considered significant.

3. Results

3.1. Interactions of nAChRα1-subunit and analog peptides with RABV

The results of the virus capture ELISA indicated that the nAChRα1-subunit peptides interact with the RABV. The torpedo derived peptide

sequences (T-32 & T-20) had more affinity compared to bovine, human and rat origin sequences. T-20 had highest affinity among the analyzed peptides. However, the α-bungarotoxin binding peptides did not interact with the RABV and hence were not included in further studies (Fig. 1A). In peptide immunofluorescence tests, amino terminal end of the peptides were labeled with fluorescent dye (FITC) and these labeled peptides were used to stain the RABV infected N2A cells. The results revealed the binding of the labeled peptides to the outer membrane of the infected cells. The fluorescence was observed in groups of cells which were infected (Fig. 1B).

3.2. Inhibitory effect of peptides on RABV infection in neuronal cells:

Increasing concentrations of peptides were tested against RABV using RFFIT. The peptides pretreated groups showed reduction in the fluorescent focus units (FFU) compared to the untreated control group indicating the inhibition of RABV in N2A cells. The highest inhibition was observed in the torpedo origin peptide (T-32) with IC$_{50}$ value of 14 ± 3.01 μM. The bovine origin peptide (C-32) had relatively lower effect (IC$_{50}$: 24 ± 2.61 μM), whereas the human sequence (H-32) showed no inhibition even at 50 μM (Fig. 2). T-20 and C-20 comparatively had similar effect in inhibiting the viral infection whereas the R-14 showed no significant inhibition (Figs. 2 & 3). In the quantitative real-time PCR, the viral gene expression was reduced in peptide pretreated RABV infected cells indicating the decreased viral load. The reduction of viral copy number was highest for torpedo origin peptides (T-32 & T-20) followed by bovine origin peptides (C-32 & C-20). The human (H-32) and rat (R-14) peptide sequences did not show significant effect on the viral gene expression compared to the control groups (Fig. 4).

3.3. Effect of peptides on the attachment of RABV to neuronal cells:

The results of the viral attachment studies revealed that the nAChRα1-subunit peptides at higher concentrations were able to inhibit the RABV adsorption on the cultured neuroblastoma cells. Without the peptide treatment, the virus attachment was highest where as it decreased as the peptide concentration was increased. At 100 μM concentration of the peptide, the adsorption was least (Fig. 5). These findings indicated that the peptide may interfere with RABV attachment and entry of viruses' into the neuronal cells thereby preventing the viral infection.

4. Discussion

Nicotinic acetylcholine receptors (AChRs) are found in the central and peripheral nervous system, in vertebrate skeletal muscles and in the electric organ of fish-torpedo (Gotti and Clementi, 2004). These receptors are pentameric complexes with different subunits. The α-subunits contains major determinants for the binding of agonists, such as the neurotransmitter acetylcholine (ACh), and antagonists, including d-tubocurarine and α-neurotoxins (Karlin and Akabas, 1995). Earlier it was found that RABV interacts with α-subunit of the AchR from torpedo
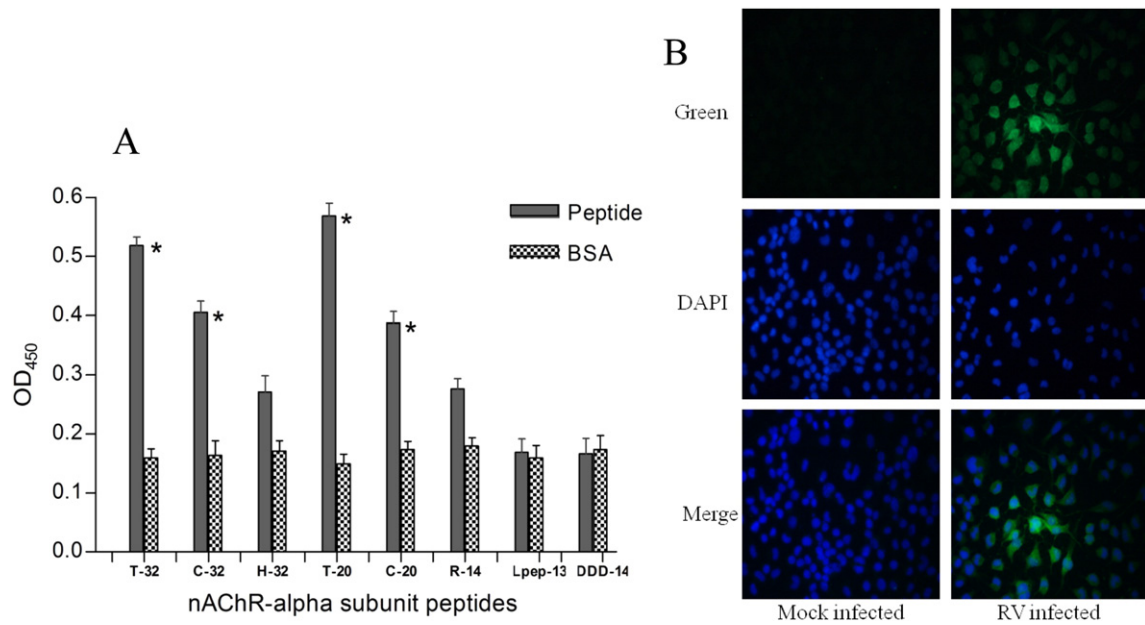
Fig. 1. nAChRα1-subunit peptide's interaction with RABV. Microtitre plates coated with 100 μg/ml of the peptides were incubated with BPL inactivated RABV. Bound virus was determined by using anti-rabies antibody, HRP-conjugated secondary antibodies and developed by TMB substrate. Binding or virus capture was determined by taking absorbance at 450 nm. For each test peptides, BSA coated control wells are included. Triplicates of two independent experiments were used for plotting the results (* indicates significant difference between the test peptide and control at p < 0.01) (A); N2A cells were infected with RABV at 0.1 moi, after 24 hpi cells were fixed, stained with FITC labeled peptides (B).

electric organ membranes and competes with bungarotoxin which binds at the residues from 173 to 204 (Gustka et al., 1996). In the present study, the nAChRα1-subunit peptide sequences of different lengths between 174 aa to 204 aa, derived from four different species (torpedo, bovine, human and rat) were analyzed for their interaction with RABV. The results revealed different affinity of peptides for RABV with T-20 showing higher binding compared to the others. Since outer coat protein, RVG has neurotoxin loop domain similar to α-bungarotoxin, the bungarotoxin binding peptides were expected to bind to RABV. However in the present study these peptides did not show any reactivity with the RABV. The comparison of the AChRα1 peptide sequences among four different species shows changes in 6
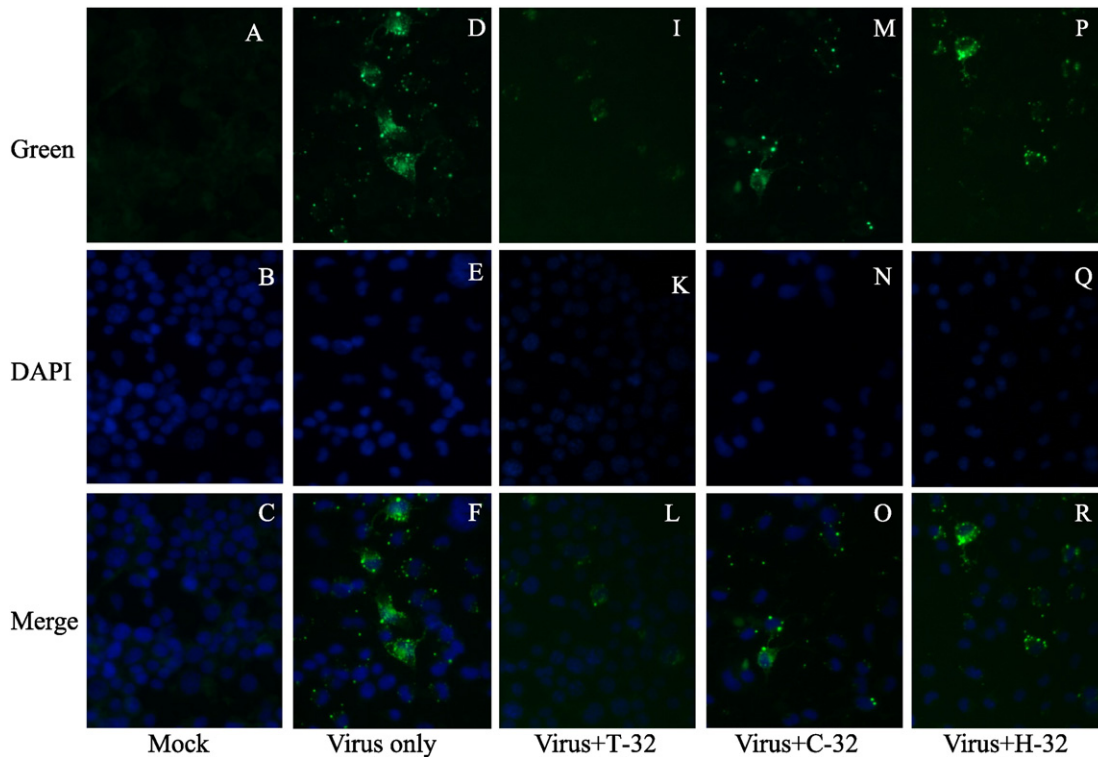

Fig. 2. Anti-RABV activity of the peptides in RFFIT in N2A cells. Experiments were carried out with the virus in the presence or absence of peptides (20 μM each). Cells were fixed after 24 hpi and stained with FITC anti-nucleocapsid conjugate. The number of cells infected were counted and compared with the positive virus control (without peptide). A, D, I, M, P — green; B, E, K, N, Q — blue; C, F, L, O, R — merge.
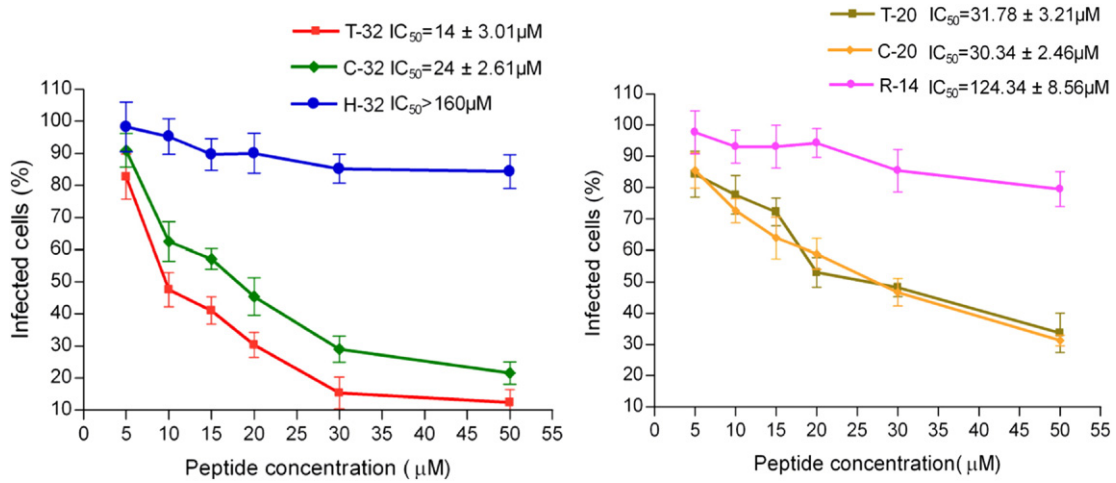
Fig. 3. Comparison of anti-RABV effect of nAChRα1-subunit peptides of different species in N2A cells based on RFFIT. RABV was pretreated with serial concentrations of peptides (0, 5, 10, 15, 20, 30 and 50 μM) for 1 h at 37 °C followed by infection on semi-confluent monolayer of N2A cells. The numbers of infected cells showing fluorescent foci were determined at 48 hpi.

residues. Between torpedo and bovine, there are two non-conservative changes (Tyr-181 to Ser and Asp-195 to Ser) whereas between T-32 and H-32 there are three non-conservative changes (Tyr-181 to Ser, Trp-187 to Ser, and Tyr-189 to Thr). In the human peptide, all the three changes involve the replacement of aromatic residues with polar residues. Considerably less affinity of the H-32 to RABV observed in our study indicates the importance of aromatic residues within these sequences. These findings were corroborated by earlier findings of α1-subunit peptide's NMR structure analysis and alanine substitutions within the peptide's sequence which also revealed a predominant contribution of aromatic and negatively charged residues in binding to the neurotoxins (Moise et al., 2002; Marinou and Tzartos, 2003).

Virus infections can be prevented by targeting the critical steps in the virus life cycle. The virus binding peptides (that are parts of host cell receptors) which specifically prevent interaction of virus glycoprotein with the host cells may block infection by preventing their attachment and entry into the cells (Teissier et al., 2011). In the present study, nAChRα1-subunit peptides were evaluated against RABV infection in N2A cells. Antiviral effect was observed when RABV was pretreated with the peptides followed by their infection in N2A cells. Previously different approaches were used to identify antiviral agents against RABV. RABV phosphoprotein (P) targeting peptides blocked

the replication of viral genome and reduced its multiplication (Real et al., 2004). Peptides mimicking the amino terminal end of P protein were found to have antiviral effect against RABV (Castel et al., 2009). Host–RABV protein–protein interactions were screened for identifying antiviral drugs against rabies (Lingappa et al., 2013). Attachment and entry of the virus are the critical steps that are targeted by antiviral peptides. nAChR α1 peptides that represent binding domain have shown to inhibit these steps in the RABV infections. Similar mechanisms of actions were involved by the peptides against other viruses including Influenza, Hepatitis C and Dengue virus, (Nicol et al., 2012; Si et al., 2012; Rothan et al., 2014). These peptides used in the present study may not be effective when the virus is already multiplying within the neurons cells as they lack cell penetrating properties. However the peptides can function when virions are freely available in extracellular conditions. It may be possible that these peptides may act similar to rabies immunoglobulin's (RIG) and prevent the virus entry to the neuronal cells.

5. Conclusions

The synthetic nAChR1-subunit peptide sequences from different species tested for interaction with RABV by virus capture ELISA and peptide immunofluorescence showed varying binding capacity with torpedo peptide exhibiting highest degree of interaction followed by bovine
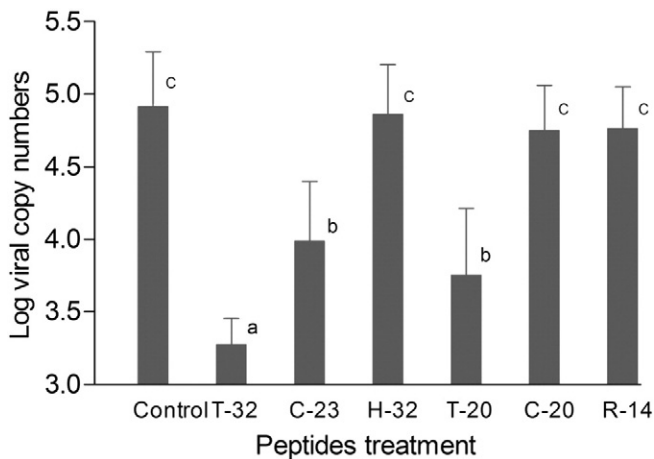

Fig. 4. Determination of viral inhibition of nAChRα-subunit peptides by qRT-PCR. The viral RNA was quantified by one step qRT-PCR at 48 hpi in RABV infected N2A cells. The viral copy numbers were determined in peptide treated (20 μM) RABV infected N2A cells. A group without peptide treatment is included as control (different superscripts on the bar indicates significant difference between groups at p < 0.01).
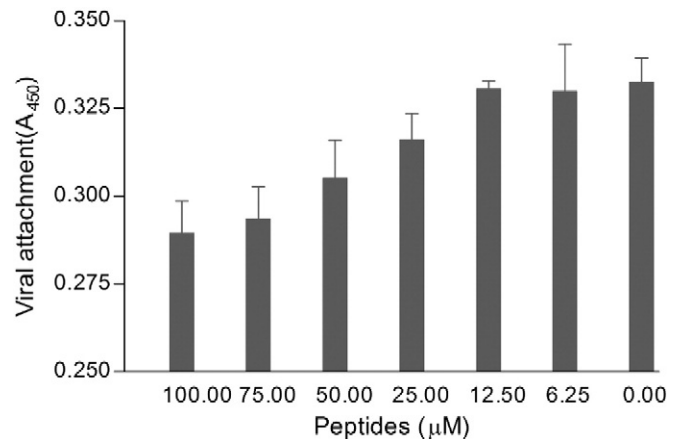

Fig. 5. Peptide mediated inhibition of virus adsorption on N2A cells. Chilled N2A cells were adsorbed with RABV previously treated with increasing concentration of T-32 peptide. Cell associated virus was detected with indirect ELISA. The values represent mean ± SEM for triplicates and representative of two independent experiments.

origin peptide sequence. These synthetic peptides inhibited the viral replication in vitro as measured by RFFIT and viral gene expression suggesting the antiviral properties of peptides. However, further in vivo studies on improved designs for higher binding capacity and ability to enter neurons are warranted to develop these peptides as potential antiviral agents for RABV and other similar viral diseases.

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Acknowledgment

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