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Brief Report

Zika E glycan loop region and Guillain-Barré Syndrome-related proteins: a possible molecular mimicry to be taken in account for vaccine development

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Abstract: Neurological complications of infection by the mosquito-borne Zika virus (ZIKV) include Guillain-Barré syndrome (GBS), an acute inflammatory demyelinating polyneuritis. GBS was first associated with recent ZIKV epidemics caused by the emergence of ZIKV Asian lineage in South Pacific. Here, we hypothesize that ZIKV-associated GBS relates to a molecular mimicry between viral envelope E (E) protein and neural proteins involved in GBS. Analysis of ZIKV epidemic strains showed that glycan loop (GL) region of the E protein includes an IVNDT motif which is conserved in voltage-dependent L-type calcium channel subunit alpha-1C ($Ca_V1.2$) and Heat Shock 70 kDa protein 12A (HSP70 12A). Both VSCC-alpha 1C and HSP70 12A belong to protein families which have been associated with neurological autoimmune diseases in central nervous system. The purpose of our *in silico* analysis is to point out that IVNDT motif of ZIKV E-GL region should be taken in consideration for the development of safe and effective anti-Zika vaccines by precluding the possibility of adverse neurologic events including autoimmune diseases such as GBS.

Keywords: ZIKV; Guillain-Barré Syndrome; Molecular Mimicry; Calcium Channel Voltage Dependent; Heat Shock Protein; Vaccine

1. Introduction

In the last decades, there has been an increasing number of epidemics associated with mosquito-borne RNA viruses of medical concern such as Dengue Virus (DENV), West Nile Virus (WNV) or Chikungunya Virus [1]. Like DENV and WNV, Zika Virus (ZIKV) belongs to flavivirus genus of the *Flaviviridae* family [2]. Classically, ZIKV is transmitted by mosquito bite and the preferred vectors for ZIKV spreading are *Aedes* genus mosquitoes [2,3]. ZIKV was first reported in Uganda in 1947 on a rhesus monkey, however the virus remained silent for years, only provoking sporadic infections until 2007 [2]. The outbreak worldwide spread urged WHO to declare ZIKV as a major public health issue, leading to a global effort to fight against the disease. ZIKV infection causes clinical manifestation in ~18% of cases ranging from mild disease with a dengue-like syndrome, to more severe outcomes such as congenital microcephaly [4–6]. Of note, other neurological disorders have been associated with ZIKV infection including encephalitis, myelitis and finally Guillain-Barré Syndrome (GBS) [5–8]. Moreover, uncommon modes of transmission have been described, including sexual and vertical (mother-to-infant) transmission, increasing the threaten that represent this virus [9–12].

Like other flaviviruses, ZIKV is a positive sense single-stranded RNA virus. The viral genomic RNA encodes a large polyprotein which is processed to generate the structural proteins C, prM and E followed by the non-structural proteins NS1 to NS5. The E protein is involved in virus binding onto host-cell and internalization of viral particles. The E ectodomain is divided into three domains I (EDI),

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II (EDII), and III (EDIII). The EDI comprises a glycan loop which is glycosylated on N154. The antibody-mediated virus neutralization depends on the availability of neutralizing epitopes on the E protein.

To date, there are two ZIKV lineages, the African lineage with the historical strain MR766 (1947) as prototype and the Asian lineage, the latter being responsible for the contemporary epidemics in South Pacific and Americas. Critically, the Asian lineage is the only one associated with GBS so far. Comparative analysis between Asian-lineage ZIKV strains PF13 (French Polynesia, 2013) and BeH819015 (Brazil, 2015) with African strain MR766 showed differences in their ability to infect human host cells [13]. The epidemic ZIKV strains differ from African strain MR766-NIID by eight amino-acid substitutions in ZIKV E protein at positions E-152/156/158/169/285/341/343. Interestingly, the residues E-152/156/158 are identified into the glycan loop (GL, residues E-151 to E-165) region of ZIKV. The GL region of Asian-lineage ZIKV strains has a glycan linked to N154 but African strain MR766 does not. We previously reported that GL region influences the availability of neutralizing epitopes on ZIKV [14] and that residues E-152/156/158 play a key role in antigenic reactivity of GL region [14]. In the present study, we proposed that amino-acids at positions E-152/156/158 of ZIKV epidemic strains might exhibit a molecular mimicry with GBS-related proteins. The possibility that E-clusters of residues E-283/285, E-341/343, and E-437/438 could account for a molecular mimicry in relation with human proteins was also investigated.

2. Materials and methods

We conducted a comparative analysis between the E sequences from epidemic Brazilian ZIKV strain BeH-819015 (Genbank access number KU365778) isolated in 2015 and laboratory-adapted African ZIKV strain MR766-NIID lineage isolated in 1947 (Genbank access number LC002520). It is of note that biological properties of viral strains BeH-819015 and MR766-NIID have been extensively studied [13–17].

We attempted to determine potential candidates for Asian lineage ZIKV E protein molecular mimicry using Blastp suite (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) included in BLAST® NCBI tool.

For this purpose, we entered as query the diverse peptides resulting from substitutions only observed in the epidemic strains of ZIKV, limiting ourselves to the small size peptides (5 amino acids). Then, we searched for protein with these sequences conserved among Human proteins (taxid: 9605) using Reference proteins database (refseq_protein) and protein-protein BLAST algorithm with default search parameters. Selection criteria for *in-silico* determination of mimicry candidates among sequences that produced significant alignment were defined as following: (i) Primary sequence fully conserved in human candidate protein; (ii) Candidate protein highly expressed in nervous system, both central and peripheric; (iii) Candidate protein near or far associated with neuropathies, in literature.

Additionally, the entire primary amino acid sequence of ZIKV (epidemic strain) envelope protein was assessed using B Cell Epitope Prediction Tools from the Immune Epitope Database (IEDB; http://tools.iedb.org/bcell/). Briefly, ZIKV E sequence was entered in the Antibody Epitope Prediction tool set on Bepipred Linear Epitope Prediction 2.0 in order to determine potential epitopes leading to antibody response in E protein. Subsequently, these results were cross-checked with the query sequence previously used for Blastp analysis.

3. Results

Molecular mimicry was proposed as a mechanism to explain how viral infection can trigger GBS [18,19]. We decided to develop an *in-silico* approach to determine whether ZIKV E protein shares homology with human proteins **(Table 1)**. So far, GBS has been associated with contemporary epidemic ZIKV strains of Asian lineage. It seems unlikely that African-lineage ZIKV have ability to mediate GBS.

Based on the amino-acid changes observed between the E proteins of viral strains BeH-819015 and MR766-NIID, we identified four clusters of amino-acid substitutions making good candidates to

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further explore molecular mimicry hypothesis **(Table 1)**. Into the EDI domain, residues at positions E-152/156/158 may play a key role in antigenic reactivity of ZIKV GL region [14]. The E-GL region of Asian-lineage ZIKV encompasses the sequence IVNDT (residues E-152 to E-156) which includes the sequon NDT where a glycan is linked to N154. African ZIKV strain MR766-NIID includes the pentapeptide TVNDI in place of IVNDT due to the (T, I) permutation at positions E-152/156 leading to a non-glycosylated ZIKV [14]. The amino-acid substitution E-Y158H has been identified between MR766-NIID and epidemic Brazilian strain BeH819015 of Asian lineage. It is of note that two substitutions at positions E-156/158 of the GL region change the pentapeptide DIGYE into DTGHE. Other amino-acid residues in the E protein are candidates for searching a sequence homology with GBS-related proteins. There are the EDII residues E-283/285 with a pentapeptide GKFLS for BeH819015 but GRLSS in MR766-NIID. Also, the EDIII residues E-341/343 compose the pentapeptide VPAQM in BeH819015 but IPVQM in MR766-NIID. Finally, the V437A and F438L permutations lead to a GALNS peptide for BeH819015 compared to GVFNS for MR766-NIID.

Query sequence	Substitutions associated	Output
IVNDT	T152I / I156T	**Calcium channel voltage-dependent L type α-1C subunit** **Heat Shock 70 kDa protein 12A** Pecanex-like protein 2 Cyclin-C Adhesion G-protein coupled receptor V1 Coagulation factor VIII HEAT repeat-containing protein 5B Sodium leak channel non-selective protein Hexokinase-1, -2, -3, HKDC1
DTGHE	I156T / Y158H	Macrophage colony-stimulating factor 1 Collagen and calcium-binding EGF domain-containing protein 1 Transcription factor COE1 Cytoplasmic FMR1-interacting protein 1 A disintegrin and metalloproteinase with thrombospondin motifs 2 Folliculin-interacting protein 1 Ubiquitin-associated protein 2-like Prolyl endopeptidase-like Adapter protein CIKS Dermokine Immunity-related GTPase family M protein Tyrosine-protein kinase ABL2 Zinc finger protein 491 Obscurin Protein prune homolog 2 Otogelin eIF-2-alpha kinase activator GCN1 BAH and coiled-coil domain-containing protein 1 Centrosome-associated protein CEP250 Supervillin Ankyrin-1
GRLSS	K283R / F285S	Pikachurin precursor Hamartin Ankyrin repeat and LEM domain-containing protein 2 Testis-expressed protein 10 GRB2-associated and regulator of MAPK protein 2 Low-density lipoprotein receptor-related protein 3 Ligand of Numb protein X 2 Synaptotagmin-like protein 4 Chromodomain Y-like protein 2

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		Oxygen-dependent coproporphyrinogen-III oxidase, mitochondrial precursor Sodium- and chloride-dependent GABA transporter 2 Acetyl-CoA acetyltransferase, cytosolic ER membrane protein complex subunit 10 Histamine H4 receptor Protein GOLM2 Protein FAM83A Uncharacterized protein C9orf163 Uncharacterized protein C3orf18 Mucin-16 Obscurin Nesprin-1 1-acyl-sn-glycerol-3-phosphate acyltransferase gamma E3 ubiquitin-protein ligase HERC2 Histone-lysine N-methyltransferase 2A Serine/threonine-protein kinase mTOR Myosin light chain kinase, smooth muscle
VPAQM	I341V / V343A	Zinc finger protein 646, 292, 831, GLI1 Galactoside alpha-(1,2)-fucosyltransferase 2 Tensin-2 Neurolysin, mitochondrial Filamin-A Spectrin beta chain, non-erythrocytic 5 Structure-specific endonuclease subunit SLX4 Adhesion G protein-coupled receptor L3 Tau-tubulin kinase 1 IQ domain-containing protein N N-acetylglucosamine-1-phosphotransferase subunits alpha/beta FERM domain-containing protein 4A RE1-silencing transcription factor Protocadherin-8 Protein transport protein Sec24D Adhesion G-protein coupled receptor G2 Protein FAN Kinesin-like protein KIF18B
GALNS	V437A / F438L	A-kinase anchor protein 12 EMILIN-1 precursor DENN domain-containing protein 1C Gelsolin Tudor domain-containing protein 10 CXXC-type zinc finger protein 4 Obscurin Microtubule-actin cross-linking factor 1 Usherin Ryanodine receptor 2 Dynein heavy chain 8, heavy chain 14, heavy chain 7 Sacsin Basement membrane-specific heparan sulfate proteoglycan core protein DNA-dependent protein kinase catalytic subunit Protein bassoon Transformation/transcription domain-associated protein Xin actin-binding repeat-containing protein 2 Protocadherin-16 precursor

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Table 1. Host proteins obtained from query sequence alignment using Blastp. The proteins highlighted in bold are the only ones which fulfill the criteria of selection as candidate for molecular mimicry.

With the aim to understand the underlying mechanism for ZIKV-mediated GBS, *in-silico* methods were employed to identify potential protein candidates for molecular mimicry. Consequently, protein sequence alignment based on the E proteins of BeH819015 and MR766-NIID was assessed with human proteins, following the criteria described in the *Materials and methods* section. From all the candidates found using this method **(Table 1)**, Calcium channel voltage-dependent L type, alpha 1C subunit and Heat Shock 70 kDa protein 12A were the only ones to fulfill all the criteria **(Figure 1)**. Indeed, the IVNDT sequence is totally conserved and these proteins are highly expressed in nervous system, both central and peripheric [20–22]. Also, it has been reported, as described below, a correlation between development of neuropathies and autoantibodies directed against both voltage-dependent Ca^{2+} channel and HSP70. Thus, these proteins are exposed to immune system and therefore to autoantibody response.

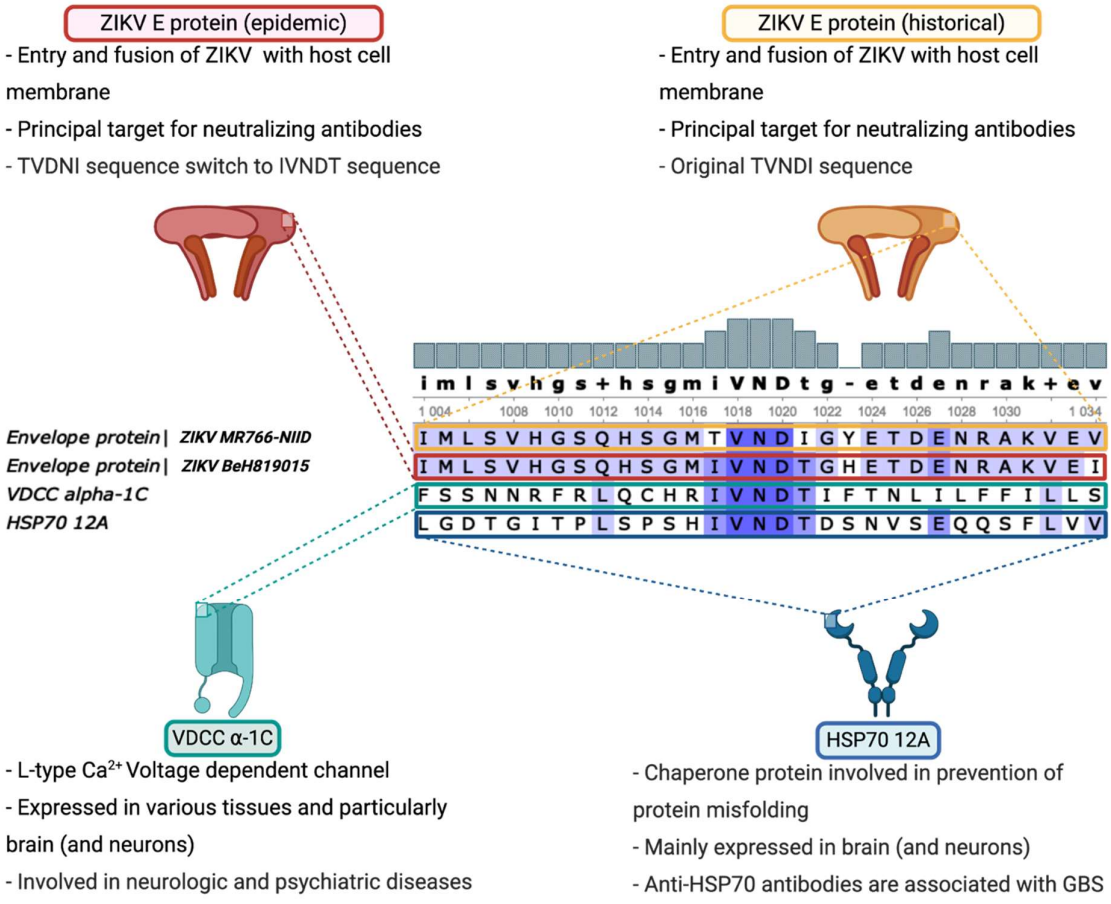

Figure 1. Calcium channel voltage-dependent L type α-1C subunit and Heat Shock 70 kDa protein 12A are potential candidates for molecular mimicry following ZIKV infection. Comparative analysis of Brazilian strain of ZIKV (BeH819015) and laboratory-adapted historical strain of ZIKV (MR766-NIID) revealed that an IVNDT polypeptide, only found in epidemic strain, might be related to ZIKV-related GBS due to Calcium channel voltage-dependent L type α-1C subunit or Heat Shock 70 kDa protein 12A molecular mimicry.

Moreover, according to antibody epitope prediction using IEDB tools, we determined that among all the predicted epitopes potentially leading to antibody response in ZIKV E sequence, only two peptides relate to regions where amino-change changes were observed in epidemic ZIKV strain

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and that served in the previously cited alignment **(Table 2)**. Strikingly, one of them contained the IVNDT sequence. Considering that other predicted antibody epitopes do not include any known amino-acid substitutions between African- and Asian-lineage ZIKV, it is highly unlikely that such E-associated epitopes may play a role in development of ZIKV-mediated GBS. Taken all together, these data form a growing body of evidence for antibody-directed against IVNDT peptide and either Calcium channel voltage-dependent L type, alpha 1C subunit or Heat Shock 70 kDa protein 12A.

Start-End	Peptide
5-9	GVSNR
66-103	SDMASDSRCPTQGEAYLDKQSDTQYVCKRTLVDRGWGN
126-133	TGKSIQPE
146-163	SQHSGM**IVNDT**GHETDEN
193-197	RTGLD
218-240	FHDIPLPWHAGADTGTPHWNNKE
274-279	EAEMDG
312-322	TFTKIPAETL
349-352	MQTL
368-371	STEN
395-408	KITHHWHRSGSTIG
428-440	AWDFGSVG**GALNS**

Table 2. Antibody epitope prediction for epidemical ZIKV (BEH819015) envelope protein. Summary of all antibody predicted epitope using IEDB tools with ZIKV (BEH819015) envelope protein as query. In **bold** are highlighted the sequences with substitutions in BEH819015 strain compared with MR766-NIID strain.

4. Discussion

Voltage-dependent calcium channels (VDCCs) are key proteins modulating Ca^{2+} entry into electrically excitable cells, following depolarization. Calcium channel voltage-dependent L type α-1C subunit, also called $Ca_v1.2$, is part of L-type VDCCs family [20]. This Ca^{2+} channel participates in hippocampal long-term potentiation, hippocampus-dependent forms of memory, peripheral vascular resistance, cardiac inotropy or insulin secretion [20,23]. Impairment of similar channels by anti-GM1 antibodies was implicated in development of GBS [24,25]. Since, anti-GM1 antibodies are found at low level in ZIKV-induced GBS patient, it is likely that voltage-dependent Ca^{2+} channel is targeted by different cross-reacting antibodies. Here, we showed the existence of an IVNDT conserved sequence between $Ca_v1.2$ and ZIKV (BEH819015) envelope protein. Even if, $Ca_v1.2$ has never been directly implicated into development of GBS, nor other neuropathy, we suggest that the recognition of $Ca_v1.2$ on neuron by cross-reacting anti-IVNDT antibodies, following ZIKV infection, might lead to initiation of immune response and development of GBS pathophysiology (**Figure 2**). However, this hypothesis remains to be tested. Since $Ca_v1.2$ is mainly involved in cardiac and memory functions, the development of symptoms associated with these functions should be expected (*e.g.* cardiac and psychiatric forms), but to date none was reported.

Heat Shock Proteins (HSP) are divided into subfamilies according to their theoretical molecular weights (27-kDa, 60-kDa, 70-kDa and 90-kDa). They are known for their crucial role in preventing protein misfolding and aggregation, as for their induction in case of cellular stress such as increased temperature radiation, exposure to chemicals, oxidative stress and various physiological and

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pathological stimuli [26]. An additional role of HSP is the participation to antigen presentation / cross-presentation [27] and inflammatory signaling [28]. Among this large family, HSP70s (to which belongs Heat Shock 70-kDa protein 12A – HSP70 12A) have been associated with autoimmune neurological disorders and more specifically immune-induced neuropathies like GBS [29]. Indeed, it has been reported an increased prevalence of anti-HSP70 antibodies in GBS patients [26,30]. Here, we showed the existence of an IVNDT conserved sequence between HSP70 12A and ZIKV (BeH819015) envelope protein. As HSP70 12A is mainly expressed in neural cells of the central nervous system (CNS), a such finding might be meaningful for ZIKV-associated GBS pathophysiology insights. Based of our molecular mimicry hypothesis, it is proposed that anti-IVNDT antibodies produced during ZIKV infection cross-react with HSP70 expressed by neurons promoting complement recruitment, membrane attack complex formation and autoimmune reaction initiation **(Figure 2**).

Our *in-silico* data allowed to propose a molecular mimicry hypothesis between the Zika E sequence IVNDT and human neuronal proteins (*e.g.* $Ca_v1.2$ and HSP70 12A) within the CNS. The pentapeptide IVNDT which composes the sequon NDT into the EDI domain of Asian-lineage ZIKV is highly conserved among contemporary epidemic viral strains. Different sub-strains of historical African strain MR766 as a ZIKV prototype have been identified as non-glycosylated ZIKV. Thus, MR766 substrain NIID (Genbank access number LC002520.1) contains a TVNDI sequence in the glycan loop region whereas M.mulatta-tc/UGA/1947/MR-766 substrain (Genbank access number KU955594) bears a 4-aa deletion leading to a lack of VNDI sequence. To our knowledge, non-glycosylated ZIKV strains have never been involved in ZIKV-mediated GBS. It is therefore tempting to propose a link between the pentapeptide IVNDT found in Asian-lineage ZIKV in relation with human proteins $Ca_v1.2$ and HSP70 12A and the development of GBS in Zika patients. Our molecular mimicry hypothesis will need to be proven with the detection of anti-IVNDT cross-reacting antibodies in GBS patients diagnosed for ZIKV infection as well as development of animal model for understanding the mechanisms by which IVNDT peptide-related antibodies could trigger GBS.

In conclusion, we propose that Zika IVNDT peptide should be taken in consideration to preclude the risks of adverse neurologic autoimmune diseases such as GBS during the course of ZIKV infection. Our molecular mimicry hypothesis raises the question of vaccine development against ZIKV. Indeed, a possible causal relationship between molecular mimicry and development of autoimmunity in response to ZIKV infection alerts on the need for vaccine candidates exempt from viral antibody epitopes identified as potential GBS triggers. We recently reported the development of a chimeric viral clone called ZIKALIVax, which was designed with viral strain MR766-NIID as backbone and structural protein region of epidemic strain BeH918015 [14]. The replacement of pentapeptide IVNDT into BeH918015 glycan loop region by TVNDI sequence from MR766-NIID resulted in a non-glycosylated envelope protein [14]. ZIKALIVax was proposed as live-attenuated vaccine candidate against ZIKV-related disease [14]. Based on our molecular mimicry hypothesis between ZIKV sequence IVNDT and human proteins $Ca_v1.2$ and HSP70 12A, it is presumed that ZIKALIVax cannot trigger GBS upon vaccination reinforcing the attractivity of a such chimeric viral clone as vaccine candidate against Zika.

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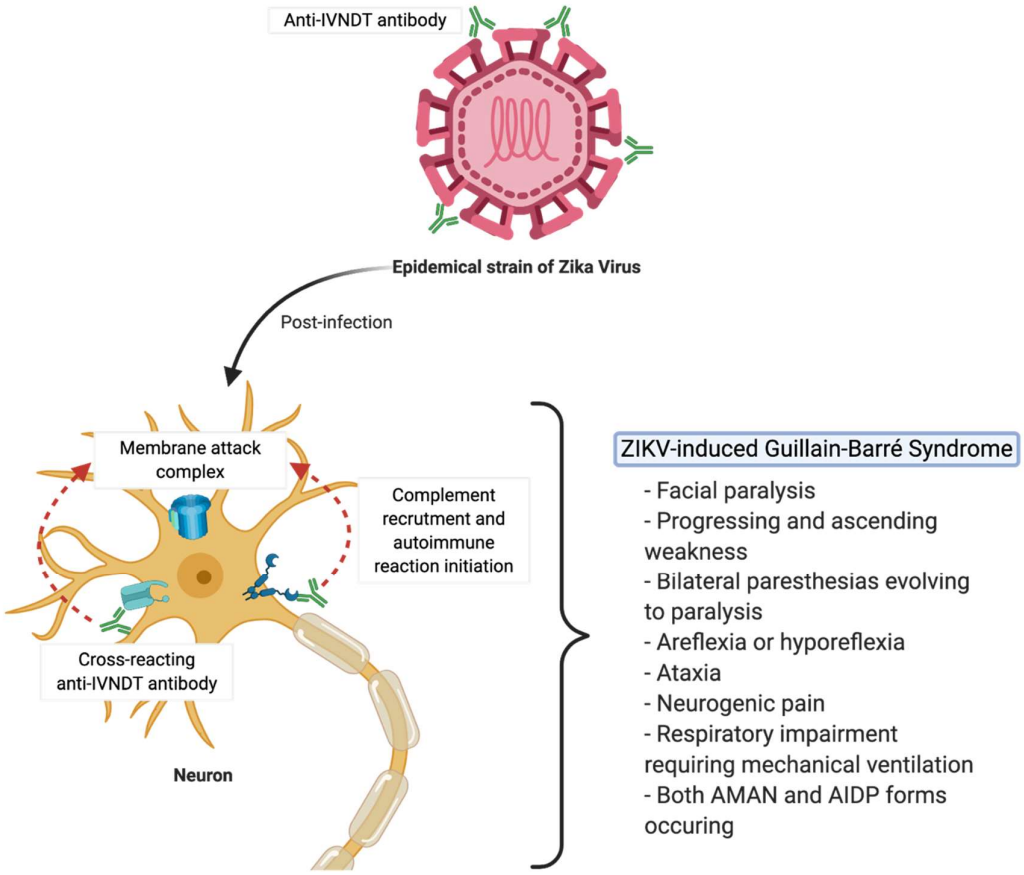

Figure 2. ZIKV-induced Guillain-Barré Syndrome might be promoted by auto-antibodies directed against Calcium channel voltage-dependent L type α-1C subunit or Heat Shock 70 kDa protein 12A.

Abbreviations

BeH819015	Clinical isolate of ZIKV collected in Brazil in 2015
$Ca_V1.2$	Calcium channel voltage-dependent L-type α-1C subunit
GBS	Guillain-Barré Syndrome
HSP70 12A	Heat Shock 70 kDa protein 12A
MR766-NIID	Laboratory-adapted ZIKV strain isolated in Uganda in 1947
VDCC	Voltage-dependent calcium channel
ZIKV	Zika Virus

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Conflicts of Interest: The authors declare no conflict of interest.

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