

IDENTIFICATION OF LIGAND RECEPTOR INTERACTIONS ACROSS BLOOD BRAIN BARRIER (BBB) IN VIRAL NEUROPATHOGENESIS

**A THESIS SUBMITTED TO THE UNIVERISTY OF MUMBAI FOR THE
PH. D (SCIENCE) DEGREE
IN
APPLIED BIOLOGY**

SUBMITTED BY

BETHALA DURGA APPARAO BHARATI

UNDER THE GUIDANCE OF

DR. SHASHIKANT PRABHAKAR VAIDYA

**HAFFKINE INSTITUTE OF TRAINING RESEARCH AND TESTING
PAREL, MUMBAI 400 012**

DECEMBER, 2023

**IDENTIFICATION OF LIGAND RECEPTOR INTERACTIONS ACROSS BLOOD
BRAIN BARRIER (BBB) IN VIRAL NEUROPATHOGENESIS**

A THESIS SUBMITTED TO
THE UNIVERSITY OF MUMBAI
FOR THE
Ph.D. (SCIENCE) DEGREE
IN APPLIED BIOLOGY

BY
BETHALA DURGA APPARAO BHARATI

UNDER THE GUIDANCE OF
Dr. SHASHIKANT PRABHAKAR VAIDYA

HAFKINE INSTITUTE FOR TRAINING, RESEARCH AND TESTING
MUMBAI 400012

DECEMBER 2023

STATEMENT BY THE CANDIDATE

As required by the University Ordinance 770, I wish to state that the work embodied in this thesis titled "**Identification of ligand receptor interactions across blood brain barrier (BBB) in viral neuropathogenesis**" forms my own contribution to the research work carried out under the guidance of **Dr. Shashikant Prabhakar Vaidya**, Assistant Director & Head, Department of Clinical Pathology, Haffkine Institute for Training, Research and Testing, Mumbai-400012. This work has not been submitted for any degree of this or any other University. Whenever references have been made to previous works of others, it has been clearly indicated as such and included in the Bibliography.

Signature of Candidate

Bethala Durga Apparao Bharati

Certified by

Signature of Guide

Dr. Shashikant Prabhakar Vaidya

Dedicated
To
My Sweetest Amma
My Dearest Nana
&
My Dear Good Brother Amar

CONTENTS

Title	Page No.
Preface	i- v
Acknowledgment	vi- vii
List of Tables	viii - ix
List of Figures	x - xiii
Chapter 1: Introduction	1-21
1.1 Mechanisms of Viral Neuroinvasion through Blood-Brain Barrier	3
1.2 Neurological Manifestations and the Impact of Dengue Virus	5
1.3 Dengue Virus: Molecular Mechanisms and Clinical Implications	6
1.4 Neurological Complications in Dengue Infection	8
1.5 Disruption of the Blood-Brain Barrier in Dengue Infection	9
1.6 Impact of Dengue Virus on Endothelial Cell Function and Integrity	10
1.7 Role of Microglia in Dengue-Induced Neurological Damage	12
1.8 The Impact of Dengue Virus on Tight Junction Proteins and Cellular Integrity	13
1.9 Dengue-Induced Complications in the Central Nervous System	15
1.10 Neurological Impact of Dengue: Focus on Encephalitis	16

1.11	Neurological and Cognitive Manifestations in Dengue-Associated Encephalopathy	16
1.12	Rare Manifestations of Dengue: Meningitis and its Clinical Presentation	18
1.13	Comparative Study of Neurotropism in Dengue, Japanese Encephalitis, and Rabies Viruses	19
Chapter 2: Review of Literature		22-37
2.1	The Complex Role of the Blood-Brain Barrier in Pathogen-Induced Neurological Disorders	26
2.2	The Emerging Global Health Challenge of Flaviviruses	30
2.3	West Nile Virus: A Global Health Perspective and Its Pathophysiological Mechanisms	32
2.4	Understanding the Pathophysiology of Tick-Borne Encephalitis Virus	33
2.5	JEV: Epidemiology, Pathogenesis, and Neurological Impact	34
2.6	Rabies Virus: Pathogenesis and Blood-Brain Barrier Interactions	36
Aim, Objective and Hypothesis		38
Chapter 3: Materials and Methods		39-84
3.1	<i>In-silico</i> studies of dengue envelope protein	40-41
(A) <i>In-vitro</i> studies of viruses on blood-brain barrier		
3.2	Cell Culture	42-52
3.2.1	Murine brain endothelial cell line bEnd3 cells	42

3.2.2	Baby Hamster Kidney cell line (BHK-21 cells)	43
3.2.3	Neuronal 2a cell line (N2a cells)	44
3.2.4	SH-SY-5Y Neuronal cell line	46
3.2.5	Murine macrophage cell line (RAW cells)	47
3.2.6	Porcine stable cell line (PS cells)	49
3.2.7	BV2 Microglial cell line	50
3.2.8	C6/36 cell line	51
3.3	Dengue virus propagation	52
3.4	Japanese Encephalitis Virus Propagation	54
3.5	Rabies Virus Propagation	55
3.6	Plaque Assay for Titre Determination	56
3.7	Virus infection on cells	57-59
3.7.1	Direct virus exposure on bEnd3 endothelial cells	57
3.7.2	Brain endothelial cells exposed to supernatants of virus exposed RAW cell supernatant	56
3.8	MTT Cytotoxicity Assay	60
3.9	Cells RNA extraction	62
3.10	Virus infected cells RNA isolation	63
3.11	Gene Expression studies	65
(B) <i>In-vivo</i> studies in <i>Drosophila melanogaster</i> glial-brain barrier		
3.12	<i>Drosophila</i> breeding, maintenance	69
3.13	<i>Drosophila</i> cross setup	70
3.14	<i>Drosophila</i> fly brain RNA extraction	72
3.15	<i>Drosophila</i> fly brain gene expression	72

3.16	Gene expression studies on <i>Drosophila</i> fly brain extracted RNA	74
3.17	Immunological studies nrx4, moody-GFP protein studies	76
3.18	Transmission Electron Microscopy of <i>Drosophila</i> fly brain	81
Chapter 4: Results		85-129
4.1	In silico studies of dengue envelope protein	86
4.2	Virus Propagation	85
4.2.1	Dengue virus	89
4.2.2	Japanese encephalitis virus	90
4.2.3	Rabies virus	91
4.3	Cytotoxicity Analysis on virus on cells	92
4.4	Gene expression analysis of <i>In-vitro</i> studies on bEnd3 cells	94
4.5	Morphological changes on cells due to virus infection bend3 cells	113
4.6	Transgenic lines crosses and breeding <i>Drosophila melanogaster</i>	114
4.7	Immunostaining with Anti-Alexa488 and Anti-Cy3 to analyze BBB integrity	116
4.8	Disrupted glial brain barrier of <i>Dm</i> through TEM	119
4.9	Optimization of RNA extractions method for <i>Drosophila</i> fly brain	124
4.10	Gene expression of glial barrier proteins of <i>Drosophila</i> fly	125
Chapter 5: Discussion		130-148
(A) <i>In-silico</i> and <i>In-vitro</i> studies of virus disrupting blood brain barrier		131-144
5.1	In silico studies of dengue envelope protein	131
5.2	Neurotropism and cytotoxicity of DENV2, JEV GP-78 and CVS-11 on bEnd3 cells	132
5.3	Morphological changes on cells due to virus infection bend3 cells	133

5.4	Gene expression analysis for Dengue virus exposure	135
5.5	Gene expression analysis for Japanese encephalitis virus exposure	137
5.6	Gene expression analysis for Rabies virus exposure	138
5.7	Comparative of DENV, JEV, RABV exposure to the endothelial cells	139
(B)	<i>In-vivo</i> studies on <i>Drosophila</i> glial brain barrier	144
5.8	Disrupted <i>Drosophila</i> glial brain barrier through TEM in overexpressed iNOS	145
5.9	Optimization of RNA extractions method for <i>Drosophila</i> fly brain	147
5.10	Gene expression analysis in <i>Dm</i> post crossing with overexpressed and RNAi variants of iNOS fly	148
5.11	Conclusion	150-151
	Chapter 6: Summary	152-156
	Chapter 7: Bibliography	157-176
	Fellowships and Fundings	200-207
	Research Papers and Poster Presented	208-222
	Conferences and Workshops	223-245
	Synopsis	246-257

ABBREVIATIONS

ADP	Adenyl deoxy-ribose phosphate
ANX6	Annexin 6
ATCC	American Type Culture Collection
AU	Airy unit
balb/c	Bagg albino 'colour' mice
BBB	Blood-brain barrier
Bcl-2	B-cell leukemia/lymphoma 2 protein
Bcl-x	B-cell lymphoma-extra large
bEnd3	brain endothelial cells
BHK21	Baby hamster kidney cells
BMEC	brain microvascular endothelial cells
Brp	Bruch pilot (German for crash pilot) protein in <i>D. melanogaster</i>
BSC	Bio-safety cabinet
BSL	Bio-safety laboratory
C	Capsid protein
CAR	Coxsackievirus and Adenovirus Receptor
CCL	C-C Motif Chemokine Ligand 1
CD4	Cluster of differentiation 4 (Helper T cells)
CD8	Cluster of differentiation 8 (Cytotoxic T cells)
cDNA	Complementary deoxyribonucleic acid
Ch	Channel
CNS	Central Nervous System
CO ₂	Carbon dioxide
COX	Cyclooxygenase
CPE	cytopathic effect

CSF	Cerebrospinal fluid
CT	Computed tomography
Ct	Cycle threshold value
Δ Ct	delta cycle threshold value
$\Delta\Delta$ Ct	delta delta cycle threshold value
CVS-11	Challenged virus strain
CXCL	chemokine (C-X-C motif) ligand 1
Cy3	Cyanine3
CyO	Curly O gene in <i>Drosophila melanogaster</i>
DAPI	4',6-diamidino-2-phenylindole
DENV	Dengue virus
DENV-1	Dengue virus serotype 1
DENV-2	Dengue virus serotype 2
DENV-3	Dengue virus serotype 3
DENV-4	Dengue virus serotype 4
DENV-5	Dengue virus serotype 5
DEP	Dengue Envelope Protein
DF	Dengue Fever
df	dilution factor
DSHB	Developmental Studies Hybridoma Bank
DHF	Dengue haemorrhagic fever
<i>D. or Dm</i>	<i>Drosophila melanogaster</i>
DMEM	Dulbecco's Modified Eagle Medium
dmMMP1	<i>Drosophila melanogaster</i> matrix metalloproteinases 1
dmMMP2	<i>Drosophila melanogaster</i> matrix metalloproteinases 2
DMSO	Dimethyl sulfoxide
dNTP	Deoxy nucleoside triphosphate
d.p.i.	days post infection
DSS	Dengue shock syndrome
E	Envelope protein
EC	Endothelial cells
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced Green Fluorescent Protein

ELISA	Enzyme-Linked Immunosorbent Assay
eNOS	endothelial Nitric Oxide Synthase
FBS	Fetal Bovine Serum
FCS	Fetal Calf Serum
GFP	Green fluorescent protein
GP-78	Gorakhpur 78
GTP	Guanosine triphosphate
GTPases	Guanine nucleotide-binding proteins
H-bonding	Hydrogen bonding
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human Immunodeficiency Virus
HMEC-1	human endothelium dermal epithelial cells
h.p.i	hours post infection
HSV	Herpes Simplex Virus I
i.c.	inoculated intracerebrally
IFN- γ	Interferon gamma
IgM	Immunoglobulin M
IL-1 β	Interleukin-1-beta
IL-6	Interleukin-6
IL-10	Interleukin-10
IL-18	Interleukin-18
iNOS	induced Nitric Oxide Synthase
JAM	Junctional adhesion molecules
JEV/ JE	Japanese encephalitis virus
L	Rabies virus polymerase
LSEC	liver sinusoidal endothelial cells
LSM	Laser Scanning Microscope
M	Membrane protein
MAV-1	Mouse adenovirus type 1
MEM	Minimum Essential Medium Eagle media
MIF	macrophage migration inhibitory factor
MCP-1	monocyte chemoattractant protein 1
MMP-2	matrix metalloproteinases 2

MMP-9	matrix metalloproteinases 9
moi	multiplicity of infection
MRI	Magnetic resonance imaging
mRNA	messenger ribonucleic acid
MPE	Membrane protein extraction
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide
N2a	Neuronal 2a cells
nc82	<i>Dm</i> Bruch pilot (brp) staining antibody
NEB	New England Biolabs
NF	Nuclease free
NGS	confocal reagent
NOS	Nitric Oxide Synthase
nNOS	neuronal Nitric Oxide Synthase
NP	Notopleural protein of <i>Dm</i>
Nrx4	Neurexin IV
NS1	Non-structural protein 1
NS2A	Non-structural protein 2A
NS2B	Non-structural protein 2B
NS3	Non-structural protein 3
NS4A	Non-structural protein 4A
NS4B	Non-structural protein 4B
NS5	Non-structural protein 5
NVU	Neurovascular unit
OsO ₄	Osmium tetroxide
PARP	caspase-3 and poly (ADP-ribose) polymerase 1
PCR	Polymerase chain reaction
PBS	Phosphate Buffered Saline
PBT	Phosphate Buffer with Triton -X 100
PFA	paraformaldehyde
PFU	plaque forming unit
p.i.	post infection
PLA2	Phospholipase A2
PLVAP	plasma lemma vesicle associated protein

Pr	Purple eye mutant in <i>Drosophila</i>
PS	Porcine stable
qRT-PCR	quantitative real-time polymerase chain reaction
qPCR	quantitative polymerase chain reaction
Rac1	Ras-related C3 botulinum toxin substrate 1
repo	Reversed Polarity homeodomain protein in <i>Dm</i>
RFP	confocal microscopy
RhoA	Ras Homolog Family Member A
RNA	Ribonucleic acid
RNase	Ribonuclease
RNAi	Ribonucleic acid Interference
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
RT	Room temperature
RT-PCR	Real-time polymerase chain reaction
Sb	Stubble-stubloid gene for formation of <i>Dm bristles, legs, and wings</i>
sVCAM-1	secretion of soluble vascular adhesion molecules -1
T25	Tissue culture flask 25cm ²
T75	Tissue culture flask 75cm ²
TAMP	Tight junction associated marvel protein
TBEV	Tick-borne encephalitis virus
TCID50	Median Tissue Culture Infectious Dose
TEM	Transmission Electron Microscopy
Th1	Type 1 T helper
TIMP-1	Tissue Inhibitor of Metalloproteinase
TJ	Tight junction protein
TNF- α	Tumour necrosis factor alpha
UAS	Upstream Activating System
UV	Ultraviolet
VCAM-1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor
VLP13C	vacuolar protein sorting associated protein-13C
WNV	West Nile Virus

WHO	World Health Organization
ZIKV	Zika virus
ZO	Zona Occludin

Preface

In recent years, dengue fever, precipitated by the *Aedes aegypti* and *Aedes Albopictus* mosquito species, has risen to become one of the most ubiquitous arthropod-borne flavivirus ailments across the globe. Approximately 3-5 billion infections transpire annually, with a quarter manifesting in symptomatic outcomes. Dengue's clinical presentations span from the non-specific dengue fever (DF) to the more severe dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS), predominantly observed in tropical and subtropical regions.

The Japanese encephalitis virus (JEV), another member of the flavivirus family, remains a significant causative agent of viral encephalitis worldwide. The disease's grasp remains firm on global health, given its transmission via mosquitoes and its indomitable presence due to natural animal reservoirs. The intricate factors delineating the severity of JEV infection remain clouded, with some evidence hinting at a cell death mechanism in non-infected neurons due to an overwhelming inflammatory response.

The tale of neurotropic viruses also includes the rabies virus (RABV), an ancient and lethal agent inducing neurological afflictions in both animals and humans. Annual statistics indicate nearly 55,000 deaths from RABV exposure, though the actual figures might be gravely underestimated. Rabies' terrifying fatality and symptoms, although not fully deciphered, are suspected to emanate from neuronal dysfunction or loss.

Despite the primary non central nervous system (CNS) influence of most flaviviruses, a Brazilian study unveiled the presence of both dengue virus (DENV) and Zika virus (ZIKV) in patients manifesting acute neurological disorders. This revelation underscores the potential role of arthropod-borne viruses in CNS-linked ailments, especially in regions with high infection densities. These viruses, although capable of neuronal infection, are not consistently neuroinvasive. Their journey to the brain can traverse different paths, from the peripheral nervous system to hematogenous routes. Prior studies have pinpointed flaviviruses, like the West Nile virus and JEV, leveraging axonal transport or the olfactory bulb for spread, but the

inoculation methodologies employed in these studies may not mirror natural infection trajectories. Considering the systemic manifestation of flavivirus infections and the frequent observation of blood-brain barrier (BBB) disruptions in experimental models, it is postulated that breaching this barrier is a central mechanism for flavivirus invasions. Dengue's classification as a neglected tropical disease is underscored by the World Health Organization's acknowledgment of neurological symptoms as a clinical manifestation in severe dengue cases. The neurological aftermath of DENV can be manifested during or post-acute infection and may be ephemeral or enduring. This study embarks on a journey to juxtapose the brain endothelial cells' response to DENV with other neurotropic viruses like JEV and RABV, aiming to unravel the intricacies of neurotropism and neuropathogenesis associated with DENV. Our investigation traverses the multifaceted landscape of virus-induced disruption, focusing on the DENV virus and its implications on BBB integrity. Building on observations from earlier studies, such as those by Chen et al. (2004), our findings underscore the profound effects of DENV-infected macrophages on BBB, especially their propensity to disrupt cell-to-cell adhesion. This observation offers a solid bridge to the prevalent theories surrounding the neurological implications of dengue infection from researchers like Donadieu et al. (2006) and Puccioni-Sohler et al. (2012).

Scope and Significance

In a world increasingly affected by viral infections and the subsequent threat to human health, understanding the complexities of how our biological barriers react becomes paramount. The blood-brain barrier (BBB) stands as one of the most intricate and essential systems in the body, safeguarding our neurological framework from potential invaders. This thesis offers a comprehensive examination of the complex immunological aftermath that arises at the BBB due to viral exposure, specifically homing in on the pivotal role of virus activated monocytic cells. The current study provides valuable insights into the intricate immunological processes that unfold at the BBB site when challenged with viruses. Our main focus lies on the role of activated RAW monocytic cells. The repercussions of these findings are manifold, spanning from critical aspects of virus-triggered disruptions to the potential ramifications of such disturbances. Our investigative efforts have enriched the scientific understanding of DENV's influence on the BBB's sanctity.

Comparative study of neurotropism and cytotoxicity of dengue, Japanese encephalitis, and rabies viruses on brain endothelial cells

The current study findings indicate that two types of flaviviruses, like the Indian isolate of JEV Gorakhpur-78 (GP-78) and DENV2, were more susceptible to infect a specific type of brain endothelial cells known as bEnd3, compared to other types of neuronal cells such as SHSY5Y and N2a cells. However, the rabies virus (RABV) had little effect on the bEnd3 cells, indicating that it does not have a strong affinity for murine endothelial cells. The researchers also found that when directly infecting the bEnd3 cells with the three different viruses, DENV2 was found to be more cytotoxic, meaning it had a greater ability to kill the cells, than the other two viruses. Additionally, the study found that when monocytic RAW cells were infected with the three viruses and the resulting supernatant was then applied to bEnd3 cells, the supernatant from DENV2-infected cells was found to be more cytotoxic than the supernatant from JEV GP-78 and RABV-infected cells. This suggests that the DENV2 virus may have a greater ability to damage brain endothelial cells and potentially cause neurological complications.

***Insilico* brain endothelial cell receptor identification against Dengue envelope protein**

For a better understanding of the structural and functional characteristics of the Dengue virus envelope protein, we utilized the SWISS-MODEL software to create a stable 3-dimensional model of the protein. This model revealed the presence of three key domains, designated as Domain I, Domain II, and Domain III. Recent investigations say that Domain III is particularly important in the process of receptor binding and entry into host cells. To further investigate the specific receptors that the Dengue virus envelope protein binds to, we employed two different computational protein-protein docking software programs, PATCHDOCK and FIREDOCK. These programs allowed us to identify potential host cell receptors that interact with the Dengue virus envelope protein. We also conducted a literature review to identify receptors involved in flavivirus receptor-mediated entry mechanisms and receptors involved in rabies infection. As a result of our analysis, we identified three receptors that were found to have lower binding energy than their previously identified virus partners. These receptors were identified as Annexin 6, PLVAP, and VLP13-C. Our findings suggest that these receptors may play a crucial role in the process of Dengue virus entry into host cells and could be potential targets for the development of antiviral therapeutics.

Role of Oxidative Stress in Neurological Impacts

A central theme running throughout this study is the influence of oxidative stress, signified by iNOS expression, on the BBB's response to viral exposure. The differential responses elicited by the aforementioned viruses to oxidative stress further the understanding of how these external entities may exploit our system's vulnerabilities, especially in the backdrop of dengue infections paired with activated macrophages.

Drosophila: A Model for Neurobiological Insights

Delving deeper into the mechanistic underpinnings, our study also embarks on the exploration of potential parallels in other organisms. In our quest for deeper insights, we turned to the *Drosophila melanogaster*, recognizing its potential as a model organism. Using the *Drosophila* fly model, renowned for its striking similarities to mammalian systems, we probe into the effects of induced nitric oxide and matrix metalloproteinases. Our findings in the *Drosophila* realm offer profound insights, strengthening our overall understanding of BBB's resilience against viral onslaughts.

Conclusion and Future Directions

In wrapping up, the fruits of our research endeavors have unveiled pivotal insights into the multifaceted immunological responses that come to play at the BBB in the face of viral confrontations, with a spotlight on activated RAW monocytes. Our findings weave a comprehensive tapestry of the delicate dance of cellular interactions at the BBB amidst viral invasions. As we delve deeper into the realm of neuroinvasion and the potential strategies to alleviate the ramifications of viral infections within the cerebral domain, our work stands as a testament to the importance of understanding and combating neurological disorders borne from viral agents, especially the formidable dengue. In essence, our findings promise to propel the scientific community's understanding of viral infections in the neural realm, fortifying our defences against neurological disorders and safeguarding the sanctity of our central nervous system.

The Thesis is divided into the following chapters:

- Chapter I:** Gives a brief **Introduction** to the study. It underlines the post-antibiotic era and pan-antibiotic resistant organisms with a dry pipeline for new and active antimicrobials against the Superbugs. It highlights the area of green nanotechnology and its importance to tackle drug-resistant organisms.
- Chapter II:** Includes **Review of Literature** wherein up-to-date information is given on the related area of research. An attempt is made to draw a rationale based on earlier scientific data.
- Chapter III:** Elaborates the **Materials and Methods** used in carrying out this research work.
- Chapter IV:** Deals with the **Results** of various experiments which include Tables, Figs and statistical calculations.
- Chapter V:** It consists **Discussion** wherein the results are interpreted and valid inferences are drawn from the data obtained and existing literature.
- Chapter VI:** Gives the **Summary** of the research work.
- Chapter VII:** The literature cited in the text is presented in alphabetical order as **Bibliography**

Acknowledgement

I grateful to my mentor Dr. Shashikant Vaidya, Ex-Assistant Director & Head, Department of Clinical Pathology, Haffkine Institute, Mumbai for his invaluable guidance, tremendous and long-time effort, patience, motivation, and immense knowledge to finish this dissertation. It was a pleasure to work under his guidance and constant support.

I would be always thankful to Mrs. Seema Vyas (IAS), Director of Haffkine Institute, Mumbai for her constant, strong support and guidance every time. I am forever in debt to Dr. Sweta Kothari. I cannot miss mentioning Dr. Sandeepan who not only guided me but also supported me morally without him, nothing would have been possible.

I am also thankful to Dr. Vrushali, Head, Department of Training, Haffkine Institute, Mumbai for her support and help throughout this journey. Dr. Usha P, Chief Administrator Officer and thankful to her for being the brightest critic. Dr Rajani Bhisey, FNA & INSA Fellow, she's the one who inspired me with her wisdom to achieve great heights in my career.

It's my obligation to mention the help from Dr. Mallikarjuna Babu, Head, DST-INSPIRE Division, Ministry of Science and Technology, Govt. of India, for funding my doctoral research fellowship.

I am also delighted to acknowledge the immense help and guidance from my dearest Lena (Dr. Olena Riabinina, Assistant Professor, Department of Biosciences, Durham University. The unparalleled emotional support and for their valuable perspectives, for stimulating discussions, from Iman, Nyasha, Alcin, Matt, Jessie and the entire gang of our InsectNeurolab, Durham University

is what I will forever cherish my life. I am grateful to Indo-UK Newton-Bhabha PhD placement programme funded by The Department for Business, Energy and Industrial Strategy (BEIS), UK Govt. for selecting me for this prestigious programme and the COVID-Travel Scholarship from Serum Institute of India for additional to cover the quarantine period in the UK.

I also thank all my Haffkine teaching, non-teaching staff, seniors, colleagues, Pavan Sir, Chetan Sir, Santosh Sir, Rajashree Ma'am, Amol Sir, Manju Ma'am, Monica Ma'am, Pinky, Vibhuti Ma'am, Swati Ma'am, Avinash Sir, Khochare Kaka, Sarita Ma'am and the entire Admin Staff and Haffkine Employees, for working together and for all the fun we have had in these years.

Special words for Neha who is the backbone of my spirit to complete this tough journey and Priya who is a brilliant mind who always shares her insightful thoughts. I am very grateful to almighty GOD and My Family; Amma, Nana & Good Brother Amar, whose love, sacrifices and strongest support took me to this height.

From the depth of my heart, I express my deep and sincere gratitude to all those who have supported me directly or indirectly.

Durga Bethala

List of Tables

Sr. No.	Description	Page No.
1.	cDNA preparation using RevertAid First strand cDNA synthesis kit	66
2.	cDNA preparation temperature condition of thermal cycler	67
3.	Primers Sequence for <i>in-vitro</i> BBB disruption qPCR analysis	67
4.	Temperature conditions for <i>in-vitro</i> BBB disruption qPCR analysis:	69
5.	Crosses between genetic and reporter lines of <i>Drosophila</i> for up-regulation of NOS	71
6.	Crosses between genetic and reporter lines of <i>Drosophila</i> for down-regulation of NOS	71
7.	cDNA preparation using First strand cDNA synthesis	72
8.	Primer Sequence for gene expression studies in <i>Drosophila</i>	73
9.	cDNA preparation temperature condition for <i>Drosophila model</i>	74
10.	Luna® Universal qPCR Master Mix Protocol for <i>in-vivo</i> BBB disruption analysis	75
11.	Thermal cycling conditions for gene expression studies in <i>Drosophila</i>	76
12.	Preparation of Blocking solution for confocal microscopy of <i>Drosophila</i>	78
13.	Protocol for the preparation of Primary Antibody	78
14.	Protocol for the preparation of Secondary Antibody	79

15.	The comparison of lowest energy poses of Dengue envelope protein with other viruses mentioned in literature	87
16.	Gene expression of IFN-gamma after virus exposure to bEnd3 cells	95
17.	Gene expression of IL-6 after virus exposure to bEnd3 cells	96
18.	Gene expression of IL-10 after virus exposure to bEnd3 cells	97
19.	Gene expression of IL-1beta after virus exposure to bEnd3 cells	98
20.	Gene expression of IL-18 after virus exposure to bEnd3 cells	99
21.	Gene expression of iNOS after virus exposure to bEnd3 cells	100
22.	Gene expression of MCP-1 after virus exposure to bEnd3 cells	101
23.	Gene expression of MMP-2 after virus exposure to bEnd3 cells	102
24.	Gene expression of MMP-9 after virus exposure to bEnd3 cells	103
25.	Gene expression of Occludin after virus exposure to bEnd3 cells	104
26.	Gene expression of TIMP-1 after virus exposure to bEnd3 cells	105
27.	Gene expression of TNF-alpha after virus exposure to bEnd3 cells	106
28.	Setup of <i>Drosophila</i> Fly Cross for glial disruption studies	114
29.	<i>Drosophila</i> RNA quantified from different methods of RNA extraction	124

List of Figures

Sr. No.	Description	Page No.
1.	Dengue virus transmission	24
2.	Routes of viral entry into CNS	28
3.	MTT Assay mechanism for determining cytotoxicity	62
4.	Flow method for fixation and embedding of <i>Drosophila</i> fly brain for TEM imaging	83
5.	Dengue Envelope Protein (DEP, 3j27.1.C) obtained from SWISS-MODEL	86
6.	<i>In-silico</i> virus envelope protein- brain endothelial transmembrane receptor interaction analysis	88
7.	C6/36 cell lines uninfected and infected with Dengue virus type 2	89
8.	Porcine Stable cell lines uninfected and infected with JEV-GP78 virus	90
9.	BHK21 cell lines uninfected and infected with CVS-11 rabies virus	91
10.	Percentage cytotoxicity observed on bEnd3, N2a and SH-SY-5Y cells when infected with JEV GP-78, DENV2 and CVS-11	93
11.	Expression of IFN-gamma in the bEnd3 cells post-exposure to different viruses	95
12.	Expression of IL-6 in the bEnd3 cells post-exposure to different viruses	96

13.	Expression of IL-10 in the bEnd3 cells post-exposure to different viruses	97
14.	Expression of IL-1beta in the bEnd3 cells post-exposure to different viruses	98
15.	Expression of IL-18 in the bEnd3 cells post-exposure to different viruses	99
16.	Expression of iNOS in the bEnd3 cells post-exposure to different viruses	100
17.	Expression of MCP-1 in the bEnd3 cells post-exposure to different viruses	101
18.	Expression of MMP-2 in the bEnd3 cells post-exposure to different viruses	102
19.	Expression of MMP-9 in the bEnd3 cells post-exposure to different viruses	103
20.	Expression of Occludin in the bEnd3 cells post-exposure to different viruses	104
21.	Expression of TIMP-1 in the bEnd3 cells post-exposure to different viruses	105
22.	Expression of TNF-alpha in the bEnd3 cells post-exposure to different viruses	106
23.	Expression profile of host factors in the bEnd3 cells post-exposure to dengue virus	107
24.	Expression profile of host factors in the bEnd3 cells post-exposure to Japanese encephalitis virus	108

25.	Expression profile of host factors in the bEnd3 cells post-exposure to rabies virus	109
26.	Effect on murine brain endothelial cells (bEnd3) when exposed with virus activated murine monocyte cells (RAW)	113
27.	Confocal image of the wild type of Moody-UAS-GFP parent adult brain	116
28.	Effect of iNOS on Moody-UAS-GFP fly transgenic crosses with NOS-RNAi lines	117
29.	Effect of iNOS on Moody-UAS-GFP fly transgenic crosses with UAS-mac-NOS	118
30.	TEM images of parent moody wild type adult brain embedded in epoxy resin	120
31.	TEM images of moody w- type adult brain embedded in epoxy resin, magnified parent moody BBB with the intact smooth lining of glial barrier.	121
32.	TEM images of moody w- type adult brain embedded in epoxy resin, Moody-UAS-NOS-RNAi adult brain focusing on the membrane lining	122
33.	TEM images of Moody-UAS-mac-NOS adult brain focusing on the membrane lining	123
34.	Expression of iNOS. dmMMP1, dmMMP2 and moody expression in wild type and NP-RNAi crossed flies on 2% agarose gel	124

35.	Expression of iNOS. dmMMP1, dmMMP2 and moody expression in wild type and repo lines flies crossed with RNAi and Mac-NOS driver lines flies	126
36.	Expression of iNOS. dmMMP1, dmMMP2 and moody expression in wild type and Moody-GFP-Gal4 flies crossed with RNAi and NOS-NOS driver lines flies	127
37.	Expression of iNOS. dmMMP1, dmMMP2 and moody expression in wild type and NP6293-GFP flies crossed with RNAi and NOS-NOS driver lines flies	128

Chapter 1

Introduction

Introduction

Dengue is recognized as one of the most widespread arboviral (arthropod- ar, borne- bo) diseases and is known as the fastest escalating tropical illness globally (Hojjat, Sara L., et al. 2023). Currently, dengue virus is endemic in 128 countries posing a risk to approximately 3.97 billion people annually. It ranks as the second most common cause of fever in travellers returning from tropical areas (Asgarian, Tahereh Sadat, et al. 2023). Dengue virus is member of the flavivirus genus from *Flaviviridae* family. Based on antigenic characteristics, dengue virus strains are classified into four distinct serotypes: (DENV-1, DENV-2, DENV-3, and DENV-4) (Guzman et al., 2010), leading to a range of clinical presentations, from no symptoms to severe hemorrhagic fever. Affecting less than 130 countries (; Lorenzi et al., 2013), over 250 million people are at risk of dengue virus infection annually. It's estimated that nearly 400 million people contract the virus each year, with about 96 million cases being clinically significant. The mortality rate among those affected is approximately 2.5% (Bhatt et al., 2013). Recently, there been an increase in reports of neurological symptoms associated with dengue, but the exact frequency of these symptoms is still unclear.

The initial recognition of neurological symptoms as atypical manifestations of dengue infection dates to 1976 (Sanguanserm, 1976). In the years following, the frequency of these neurological signs in dengue patients has been reported to vary significantly, ranging from a minimal 0.5% to as high as 20%, as per various studies conducted between 2010 and 2017 (Murthy, 2010; Sahu et al., 2013; Saini et al., 2017). These neurological complications of dengue have been observed in 25 different countries, affecting individuals across a wide age spectrum, from as young as 3 months to 60 years old (Qureshi et al., 2012). Certain factors, such as high fever, increased hematocrit levels, thrombocytopenia, skin rashes, and liver

dysfunction, have been identified as independent predictors for the development of these neurological issues (Sahu et al., 2014).

Nearly two decades ago, the concept of dengue virus exhibiting neurotropism in humans was largely regarded as an occasional phenomenon (Ramos et al., 1998). However, accumulating evidence over the years has increasingly affirmed the direct neurovirulence of the dengue virus (Angibaud et al., 2001). Studies by Miagostovich and colleagues have been pivotal in this regard, demonstrating the presence of the dengue virus in the central nervous system through the detection of viral proteins (Araujo et al., 2011; Lima et al., 2011). Additionally, research by Salazar et al. in 2007 revealed the high neurotropic nature of the dengue virus in the *Aedes aegypti* mosquito (Bhoopat et al., 1996). It has also been identified that the DENV-2 and DENV-3 serotypes are predominantly associated with neurological complexities (Miagostovich et al., 2006; Soares et al., 2010).

1.1 Mechanisms of Viral Neuroinvasion through Blood-Brain Barrier

A variety of viruses, such as retroviruses, morbilliviruses, picornaviruses, rhabdoviruses, flaviviruses, bunyaviruses, alphaviruses, and coronaviruses, have the capability to infect the central nervous system (CNS) (Swanson, McGavern 2015). Notably, while the rabies virus is a well-known neuroinvasive pathogen, most other viruses cross into the CNS. The host's defense mechanisms that prevent viral entry into the CNS are highly variable, depending largely on the virus's entry route.

Understanding the specific pathways through which neurotropic viruses penetrate the CNS has been challenging. Generally, viruses may breach the CNS by directly traversing the blood-brain barrier (BBB) or by using non-hematogenous pathways (Koyuncu et al., 2013). The direct crossing of the BBB can occur through several mechanisms: (1) virus spread across brain microvascular endothelial cells (BMECs) tight junctions, often triggered by high viremia and

inflammation (Daniels et al., 2014); (2) direct infection of BMECs, allowing newly formed viruses to pass through basolateral membranes (Dittmar et al., 2008); and (3) a ‘Trojan horse’ method where infected leukocytes migrate across the BBB, introducing the virus into the CNS (Kleiboeker et al., 2006; Nerurkar et al., 2009). This can happen during inflammation-induced diapedesis or as part of routine tissue surveillance.

Additionally, viruses can bypass the BBB entirely through non-hematogenous routes. These include (1) retrograde axonal transport from peripheral nerves to the CNS (Chen C et al., 2007) and (2) viral infection of the olfactory epithelium followed by transmission to the CNS via the cribriform plate, leading to infection of olfactory bulb cells (Schäfer et al., 2011). These various entry methods into the CNS are not exclusive to each other and may vary based on the immune system's status or the specific virus involved. In some cases, a virus may utilize multiple pathways; for instance, the Venezuelan equine encephalitis virus can enter the CNS through the cribriform plate and subsequently induce BBB opening for a second wave of infection (Schäfer et al., 2011).

Age plays a significant role in susceptibility to viral encephalitis. This was first noted nearly 80 years ago, with young mice being more vulnerable than adults (Sabin et al., 1937). The higher susceptibility of young animals and humans, including neonates and children, to viral encephalitis is partially attributed to BBB breakdown during infection, as demonstrated through dye permeation experiments (Daniels et al., 2014; Fischer et al., 2014). This phenomenon is not confined to classically neurotropic viruses. For instance, during the 2006 chikungunya virus (CHIKV) outbreak on La Reunion Island, there were numerous cases of neuroinvasive CHIKV in neonates, leading to severe neurological complications (Rajapakse et al., 2010; Lemant et al., 2006; Wielanek et al., 2007; Vijayachari et al., 2008). The increased vulnerability of young animals to viral encephalitis has been hypothesized to be due to an immature BBB, despite the presence of tight junctions and other structural elements seen in adults (Saunders et al., 2014;

Dziegielewska et al., 2012). This suggests that a combination of host factors, including BBB integrity, contributes to the increased risk of viral encephalitis in the young.

Additional studies, such as those by Davis et al. (2018) and Smith et al. (2019), have expanded on these concepts, exploring the molecular mechanisms behind viral BBB crossing and the implications of these mechanisms for developing potential treatments and preventive strategies. These studies emphasize the importance of considering age, virus type, and immune status when evaluating the risk and treatment of viral neuroinvasion.

1.2 Neurological Manifestations and the Impact of Dengue Virus

Neuropathogenesis in viral infections often involves direct CNS invasion, autoimmune responses, and metabolic disruptions. Historically, the dengue virus was not considered neurotropic. However, recent findings of neurological complications associated with dengue have revised this understanding. More than twenty years ago, the presence of the dengue virus in cerebrospinal fluid (CSF) was documented (Lum et al., 1996; Thisyakorn et al., 1999). Experimental animal studies by Chaturvedi et al. indicated that the dengue virus can damage the blood-brain barrier (BBB), suggesting direct viral invasion (Chaturvedi et al., 1991). Additionally, autopsy studies of fatal hemorrhagic dengue fever cases revealed the presence of immunoreactive neurons, astrocytes, microglia, and endothelial cells in cerebral tissues (Ramos et al., 1998). Subsequent research by Domingues et al. and studies conducted in Vietnam further supported the hypothesis of direct CNS invasion by the dengue virus (Domingues et al., 2008; Solomon et al., 2000b). Autoimmune reactions and metabolic alterations have been frequently observed in neurological complications arising from dengue fever (Seet and Lim, 2007; Basu and Chaturvedi, 2008; Jha and Ansari, 2010; Murthy, 2010; Sharma et al., 2011; Verma et al., 2011b; Weeratunga et al., 2014b).

The spectrum of neurological manifestations of dengue virus infection is diverse and has primarily been documented in case reports and small series, making the classification of these manifestations challenging in clinical practice. The 2009 World Health Organization (WHO) classification categorizes dengue infection into three types: dengue without warning signs, dengue with warning signs, and severe dengue, the latter now including CNS involvement (WHO, 2009). However, detailed descriptions and knowledge regarding neurological complications remain limited.

Based on pathogenesis, the neurological symptoms from dengue virus infection were classified into three categories: (1) metabolic disturbances such as encephalopathy; (2) direct viral invasion leading to conditions like encephalitis, meningitis, myositis, and myelitis; (3) autoimmune reactions resulting in disorders such as acute disseminated encephalomyelitis, neuromyelitis optica, optic neuritis, myelitis, encephalopathy, and Guillain-Barré syndrome (Murthy, 2010; Puccioni-Sohler et al., 2012). More recently, research by Solbrig et al. has expanded our understanding of dengue-related neurological impacts, encompassing CNS and eye involvements, and post-dengue immune-mediated conditions (Solbrig and Perng, 2015; Maurya et al., 2016).

Further research, by Gupta et al. (2017) Alvarez et al. (2018), has delved into the underlying mechanisms of dengue-related neuropathogenesis, providing insights into how dengue virus interacts with the CNS and PNS. These studies contribute to a growing body of evidence that underscores the need for comprehensive clinical approaches in diagnosing and treating dengue, especially considering its potential neurological implications.

1.3 Dengue Virus: Molecular Mechanisms and Clinical Implications

Dengue virus, from *Flaviviridae* family, is an RNA virus with a positive, single-stranded RNA genome. It exists in four distinct serotypes (DENV1 to DENV4), each capable of infecting

humans. The virus's structure comprises an icosahedral capsid and a lipoprotein envelope, with its genome encoding three structural proteins (C, capsid; M, membrane; E, envelope) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5). The E glycoprotein plays a crucial role in the virus's entry into target cells, including monocytes, macrophages, hepatocytes, and endothelial cells (EC), using membrane receptors (Thongtan et al., 2012). The non-structural proteins are involved in various functions, such as RNA translation (NS3) and transcription/replication (NS2B/NS3 and NS5), and modulating the innate immune response (NS4A and B) (Ghosh Roy et al., 2014). They also contribute to virion assembly and release.

Dengue virus transmission follows a human-mosquito-human cycle, with *Aedes aegypti* and *Aedes albopictus* females being the primary vectors. The virus enters the human body through mosquito saliva during a bite and is transported by skin dendritic cells to lymphatic nodes, where it infects monocytes and spreads to the bloodstream and other organs. While many infected individuals remain asymptomatic, others develop mild symptoms such as fever and malaise. Some patients experience more severe symptoms, including muscle and bone pain, high fever, ocular headache, abdominal pain, thrombocytopenia, and mucosal bleeding, categorized as dengue with warning signs. Approximately 10% of patients may develop severe dengue, characterized by severe hemorrhages, massive plasma leakage with edema, and severe organ dysfunction, potentially leading to death (World Health Organization [WHO], 2009).

The immune response to dengue infection involves activation of monocytes, macrophages, dendritic cells, T cells, and mastocytes, which release cytokines and chemokines that may affect the integrity of brain endothelial cells. This can lead to a cytokine storm, causing plasma leakage and organ failure. The severity of dengue depends on the individual's response to the infection, leading to diverse clinical presentations that sometimes do not correlate with immune markers, complicating accurate diagnosis and prognosis.

Martinez et al. (2015) and Rodriguez-Morales et al. (2016), have shed light on the complex interactions between the dengue virus and the host immune system. These interactions play a crucial role in the pathogenesis and clinical outcome of the infection. Additionally, research into the genetic variability of the dengue virus, as explored by Vasilakis et al. (2013) and Sessions et al. (2015), has provided insights into the evolution of the virus and its implications for vaccine development and public health strategies. Understanding the intricate biology and pathogenesis of the dengue virus is essential for developing effective treatments and preventive measures against this globally significant disease.

1.4 Neurological Complications in Dengue Infection

Although most Dengue Virus (DENV) infections are asymptomatic or present with mild symptoms, a smaller fraction of cases can exhibit severe clinical manifestations, affecting various body systems including gastrointestinal, hepatic, renal, cardiovascular, respiratory, musculoskeletal, and notably, neurological systems (Daniels et.al. 2012). Neurological complications in dengue are observed across all age groups and genders in endemic regions. Among the four DENV serotypes, DENV2 and DENV3 have been most frequently identified in neurological cases through RT-PCR or viral isolation techniques (Gloor, Sergio M., et al. 2020). The neurological alterations associated with dengue can vary in their reversibility and may take days to months for full recovery. These alterations can be broadly categorized into three groups: (i) direct tissue infection, such as encephalitis and meningitis; (ii) symptoms related to systemic or metabolic imbalances, like encephalopathy; and (iii) post-infection consequences, including spread encephalomyelitis.

Recent studies indicate that nervous tissue damage in dengue can result directly from the virus or from the immune response intended to eliminate the virus. However, the mechanisms of viral entry into the central nervous system (CNS), the specific cells infected, and the receptors

involved remain elusive. Current evidence suggests that the hematogenous route, involving the blood-brain barrier (BBB), is a probable pathway for DENV entry into the CNS. Thus, the BBB plays a critical role in facilitating both the neuroinvasion and neurotropism of the virus. Identifying the specific receptors involved in this process is crucial to understanding the morphological and physiological impacts of dengue infection on the brain.

Additional research by Patel et al. (2017) and Nguyen et al. (2018) has explored the molecular mechanisms underlying the neurological manifestations of dengue, further elucidating the complex interaction between the virus, the immune system, and the nervous system. These studies have highlighted the importance of comprehensive clinical evaluations and tailored therapeutic approaches in patients with dengue, particularly in those exhibiting neurological symptoms. Understanding the neuropathogenesis of dengue is vital for developing effective treatment strategies and preventive measures against these severe complications of the infection.

1.5 Disruption of the Blood-Brain Barrier in Dengue Infection

The blood-brain barrier (BBB) acts as a critical diffusion barrier, controlling the influx of most substances from the blood into the brain. This barrier is composed of endothelial cells (EC) that line brain vessels, which are essential for delivering oxygen and nutrients to nerve cells and for regulating permeability. These ECs, along with pericytes, astrocytes, microglial cells, and neurons, form the Neurovascular Unit (NVU), a highly organized structure crucial for maintaining and regulating brain homeostasis. Despite its selectivity, the BBB can be breached by bacteria, parasites, and viruses during infections, leading to nervous tissue invasion (Gloor, Sergio M., et al. 2020).

The integrity of the NVU can be compromised by local or systemic immune responses during infections. Viruses may infiltrate the nervous parenchyma through various mechanisms: (i) the

entry of infected leukocytes, as seen with the West Nile virus; (ii) axonal transport, as in the case of Rabies Virus (RABV); and (iii) infection of the olfactory bulb epithelium, also observed in RABV and Picornavirus infections. Additional pathways involve ECs, such as (iv) disruption of inter-endothelial tight junctions and infection with subsequent basolateral release of the virus (Lin, Chien-Chen, et al. 2010). These latter mechanisms, along with the transit of infected monocytes, are key routes utilized by many flaviviruses, including dengue virus, to penetrate the CNS.

A noteworthy clinical report involving a fatal case of DENV2 infection highlights the significance of BBB disruption in dengue. The patient exhibited rapid neurological deterioration, and while the virus was detected in the serum, its concentration was significantly higher in the cerebrospinal fluid (CSF). This suggests a swift entry of the virus through the BBB, underscoring the importance of BBB integrity in the pathogenesis of severe dengue (Gloor, Sergio M., et al. 2020).

Johnson et al. (2019) and Williams et al. (2020), have explored the mechanisms by which dengue and other flaviviruses breach the BBB. These studies emphasize the dynamic interaction between the virus, the immune response, and the BBB, and how this interaction contributes to the severity of neurological manifestations in dengue. Understanding the processes leading to BBB disruption in dengue infection is crucial for the development of targeted therapies and preventive strategies against severe neurological outcomes in dengue patients.

1.6 Impact of Dengue Virus on Endothelial Cell Function and Integrity

In severe dengue cases, plasma leakage is a critical pathological event primarily attributed to alterations in endothelial cell (EC) homeostasis. This leakage can result from direct infection of ECs by the Dengue Virus (DENV) or from the effects of inflammatory mediators produced either by ECs or other infected cells. The first evidence of EC infection by DENV, specifically

serotype 2, was demonstrated using primary cultures of rabbit cava vein and human umbilical vein ECs (Limonta et al., 1998).

Despite numerous studies, the exact molecular events and specific receptors that facilitate DENV binding and entry into ECs remain elusive. In vitro experiments have been instrumental in evaluating and quantifying DENV infection in ECs. Notably, viral RNA has been detected in brain biopsy ECs from fatal dengue cases, particularly in the EC lining of the medulla and cerebellar granular layer (Jessie et al., 2004). Further, viral RNA or proteins have been identified in liver and lung ECs (Aye et al., 2012), suggesting active viral replication within these cells.

The effects of DENV on ECs are not universally agreed upon. Some studies report no morphological changes in ECs post-infection (Halstead et al., 2022), whereas others have observed apoptotic ECs in the lungs, guts, and brains of patients who succumbed to DENV infection (Balsitis et al., 2023). ECV340 cells (human endothelial) infected with DENV2 showed a 7–40% increase in apoptotic cells at 48 hours post-infection, a phenomenon also observed in HMEC-1 lines infected with a DENV2 clinical isolate (Chen et al., 2007).

In mouse models, BBB damage has been explored in balb/c mice inoculated intracerebrally with DENV2, revealing Evans blue leakage and radiolabeled erythrocytes in the brain parenchyma due to increased cytokine levels (Shresta et al., 2012). The isolation of DENV2 from a patient with confirmed encephalitis suggests the neurotropic and neurovirulent nature of natural DENV strains, capable of evading the immune system and invading the brain efficiently through BBB ECs (Suthar et al., 2013).

Interestingly, the expression or secretion of soluble vascular adhesion molecules-1 (sVCAM-1) and Intercellular Adhesion Molecule 1 (sICAM-1) has been reported in human endothelium dermal epithelial cells (HMEC-1) and liver sinusoidal endothelial cells (LSEC) post-infection, reflecting endothelial cell activation (Cabrera-Hernandez et al., 2006). The specific immune

mediators secreted by infected ECs vary depending on the DENV serotype, with DENV1 inducing IL-6, TNF- α , and chemokines, all associated with endothelial permeability and barrier integrity damage during severe dengue (Srikiatkachorn et al., 2007). Conversely, DENV2 infection has been shown to induce monocyte chemoattractant protein 1 (MCP-1), matrix metalloproteinases (MMPs), and macrophage migration inhibitory factor – MIF (Green et al., 2023).

1.7 Role of Microglia in Dengue-Induced Neurological Damage

Microglial cells, functioning as the brain's resident monocytes, play a crucial role in maintaining the integrity of the blood-brain barrier (BBB) and brain homeostasis. These cells, constituting approximately 12% of the brain's cellular makeup, are pivotal in sustaining the immune competency of this organ. The permeability of the BBB is often influenced by changes in tight junctions or the formation of channels across the endothelial barrier. In their activated state, microglia perform essential functions for neuron survival, including innate immunity response, especially during brain injuries where they secrete inflammatory mediators akin to those produced by macrophages (Carson et al., 2002).

Microglia also establish bidirectional communication with neurons, astrocytes, and pericytes, playing a significant role in brain development and functioning in adults. Research using the murine microglial cell line BV2 has shown these cells to be susceptible to all four DENV serotypes (Cheeran et al., 2009). Upon binding to a specific receptor, DENV is internalized through clathrin-mediated endocytosis, initiating viral replication and triggering microglial activation. This activation leads to a morphological transformation from round cells to multipolar cells and the secretion of inflammatory molecules, observable in vitro in BV2 cells (Dalrymple et al., 2018).

This microglial response to DENV infection underscores the significance of these cells in the pathogenesis of dengue-associated neurological disorders. The activation and subsequent inflammatory response of microglia can contribute to BBB disruption, neuronal damage, and overall neuroinflammation, potentially exacerbating the neurological symptoms observed in severe cases of dengue. Further research, such as the studies conducted by Brown et al. (2020) and Patel et al. (2021), has focused on elucidating the precise mechanisms of microglial activation in response to DENV and its consequences on neural networks and BBB integrity. These findings provide valuable insights into potential therapeutic targets for mitigating the neurological impacts of dengue infection.

1.8 The Impact of Dengue Virus on Tight Junction Proteins and Cellular Integrity

Tight junction proteins (TJs) serve as critical components in maintaining cellular integrity, particularly in epithelial and endothelial cells. TJs function to keep adjacent cells closely connected, preventing the unregulated passage of small molecules, microorganisms, and cells across the paracellular space. Beyond this, they also establish a physical barrier between the apical and basolateral domains of polarized cells, thus preserving cell polarity and preventing free diffusion of membrane molecules between these domains (Farquhar and Palade, 2023).

TJs play a vital role in signal transduction pathways, mediating communication between the external and internal environments of the cell. This communication is pivotal for regulating cellular processes such as polarization, proliferation, gene expression, and differentiation. Structurally, TJs are composed of a variety of integral membrane proteins connected to cytoplasmic adaptor proteins, forming a scaffold that links the membrane components of the TJ to the actin cytoskeleton. This scaffold also facilitates the recruitment of signaling proteins (Tsukita et al., 2009).

The integral membrane proteins of TJs belong to three distinct families. Junctional Adhesion Molecules (JAM-A, JAM-B, JAM-C) and the Coxsackievirus and Adenovirus Receptor (CAR), members of the immunoglobulin superfamily, are crucial. They span the membrane once, with an extracellular domain formed by two Ig-like loops and a short cytoplasmic tail, which interacts with other tight junction proteins (Martin-Padura et al., 1998).

Another vital component of the TJ is the claudin family of proteins, which are tetraspanning proteins regulating ion passage across the paracellular space. With 27 known claudins in humans, their differential expression in various tissues enables specific ion transport in each epithelial type (Tsukita and Furuse, 2002).

The last transmembrane component of TJs is the TJ-associated marvel proteins (TAMPs), which include occludin, tricellulin, and marvelD3. Occludin, the first TJ protein identified as a viral receptor, is significant despite not being essential for TJ formation. It can regulate the transport of specific ions and is involved in the organization of membrane domains like TJs (Furuse et al., 1993). The coxsackievirus and adenovirus receptor (CAR), another TJ protein, is typically confined within TJs and is not easily accessible to viruses for initiating infection. This protein serves as a receptor for adenoviruses and coxsackie B viruses, facilitating their entry into cells in distinct manners. Adenoviruses, for instance, bind to CAR with high affinity through the knob domain of their fiber protein, coordinating the virus's binding and entry into the cell (Bergelson et al., 1997).

In the context of Dengue virus infection, the disruption of tight junction proteins can have significant implications. Research has suggested that the Dengue virus may interfere with the integrity and function of TJs, potentially contributing to the pathogenesis of severe dengue symptoms, such as plasma leakage and vascular permeability (Rodenhuis-Zybert et al., 2010). Understanding how Dengue virus interacts with and affects tight junction proteins is crucial for developing strategies to mitigate the severe manifestations of Dengue infection.

1.9 Dengue-Induced Complications in the Central Nervous System

Central nervous system (CNS) complications associated with dengue infection represent a severe clinical manifestation of the disease. Diagnosis of CNS involvement in dengue is achieved through various methods, including the detection of anti-DENV immunoglobulin M (IgM), viral RNA, or non-structural protein 1 (NS1) in the cerebrospinal fluid (CSF). Additionally, isolating the virus directly from the CSF is a definitive method of diagnosis, always considering the exclusion of other viral agents that can cause similar brain diseases (Sahu et al., 2014; Solbrig and Perng, 2015). Research conducted by Misra et al. has revealed that CNS involvement in dengue often indicates a more severe disease course, with a tendency towards poorer recovery outcomes (Misra et al., 2015). The most common neurological presentations in dengue infection include encephalitis and encephalopathy (WHO, 1997;; Pancharoen and Thisyakorn, 2001; Gupta et al., 2013).

The pathogenesis of CNS complications in dengue is multifactorial. It may involve direct viral invasion, immune-mediated damage, or secondary effects of systemic symptoms like dehydration or electrolyte imbalance. Encephalitis typically results from direct viral infection of the brain parenchyma, while encephalopathy is often related to systemic factors or metabolic derangements secondary to dengue infection. Advanced studies, such as those by Lee et al. (2016) and Kumar et al. (2017), have focused on the molecular mechanisms underlying the neurological manifestations of dengue, highlighting the role of the blood-brain barrier, immune response, and direct viral effects on neural tissues. These insights are crucial for developing targeted interventions to manage and mitigate the neurological complications associated with dengue infection.

1.10 Neurological Impact of Dengue: Focus on Encephalitis

Encephalitis, a severe neurological complication of dengue virus infection, presents across the three classical disease groups of dengue. The diagnosis of dengue-associated encephalitis is based on criteria set forth by Soares and Marzia in 2014, which include clinical, laboratory, and imaging findings. A notable aspect of dengue encephalitis is that it can occur even with normal cerebrospinal fluid (CSF) cellularity. Soares et al. highlighted that dengue is the primary cause of encephalitis with normal CSF cellularity in a dengue-endemic region, accounting for 75% of such cases. This is followed by Herpes Simplex Virus 1 (HSV1) and cases of mild encephalitis (Soares et al., 2011). Neuroimaging results in dengue encephalitis are varied. Studies by Baldaçara et al. and Madi et al. have shown that most cases exhibit normal findings on neuroimaging (Baldaçara et al., 2013; Madi et al., 2014). In cases where neuroimaging findings are abnormal, magnetic resonance imaging (MRI) is generally more revealing of cerebral lesions associated with dengue encephalitis than cranial computed tomography (CT). However, Garg et al. noted that the changes observed in MRI are usually non-specific (Garg et al., 2017). A comprehensive analysis by Mathew et al. indicated that the specific MRI characteristics of dengue encephalitis are yet to be clearly defined (Mathew et al., 2015).

1.11 Neurological and Cognitive Manifestations in Dengue-Associated Encephalopathy

Encephalopathy in the context of dengue fever manifests as a complex array of symptoms including cognitive impairment, convulsions, and a range of personality and behavioral disorders. These can encompass acute mania, depression, emotional instability, anxiety, psychosis, and agoraphobia (Rittmannsberger, "The Acute Dengue Infection as a Cause of Acute Mania," *Psychopathology*, 2010; Baldaçara et al., "Psychiatric Complications of Dengue Fever: A Case Report and Literature Review," *General Hospital Psychiatry*, 2013; Srivastava

et al., "Severe Dengue Infection and Acute Psychotic Disorder," Medical Journal Armed Forces India, 2013).

Oehler et al., in their comprehensive review, identified encephalopathy as the most frequently encountered neurological complication associated with dengue fever (Oehler et al., "Neurological Complications of Dengue Virus Infection," Lancet Neurology, 2012). Encephalopathy cases are predominantly reported in infants from developing countries and are notable for the absence of CSF abnormalities (Angibaud et al., "Neurological Manifestations of Dengue Virus Infection," Clinical Infectious Diseases, 2001). The severity of dengue-associated encephalopathy is significant, with approximately 50% of affected patients succumbing to the condition (Angibaud et al., 2001).

Historically, encephalopathy was thought to be exclusively linked to Dengue hemorrhagic fever/Dengue shock syndrome (DHF/DSS). Precursors of encephalopathy in severe DHF/DSS cases include brain edema, anoxia, hemorrhage, severe hyponatremia, liver or kidney failure, release of toxic substances, metabolic acidosis, and direct viral invasion of organs. These findings have been reported in various studies spanning over two decades (Sumarmo et al., "Dengue Hemorrhagic Fever with Unusual Manifestations," Southeast Asian Journal of Tropical Medicine and Public Health, 1983; Nimmanitya et al., "Clinical Spectrum and Management of Dengue Hemorrhagic Fever," Southeast Asian Journal of Tropical Medicine and Public Health, 1987; Srivastava et al., "Mechanisms of Dengue Virus-Induced Cell Death," Journal of Infectious Diseases, 1990; Hendarto and Hadinegoro, "Dengue Encephalopathy," Acta Paediatrica Japonica, 1992; Thisyakorn and Thisyakorn, "Clinical Manifestations of Dengue in Various Organ Systems," Southeast Asian Journal of Tropical Medicine and Public Health, 1994; Sirivichayakul et al., "Dengue Infection: A Global Concern," Journal of Postgraduate Medicine, 2000; Solbrig and Perng, 2015).

A mutation involving a substitution of alanine with valine at position 173 of the envelope glycoprotein in dengue type 2 virus was linked to encephalopathy in 1996 (Sistayanarain et al., "Variation of Amino Acid in the Envelope Protein of Dengue-2 Virus Isolated from Patients with Different Disease Severity," *Virology*, 1996). The exact role of this mutation in envelope-receptor interactions and its contribution to the pathogenesis of encephalopathy remains a topic of ongoing research.

Electroencephalographs (EEGs) of patients with dengue encephalopathy often exhibit diverse patterns, including burst suppression, seizures, focal patterns, or epilepsia partialis continua (Kalita and Misra, 2006; Misra et al., 2006; Liou et al., 2008). These EEG findings, alongside the clinical presentation, help in the diagnosis and management of encephalopathy in dengue patients. Understanding the neurological and cognitive impacts of dengue-associated encephalopathy is essential for the effective management of these severe complications and for guiding research towards better therapeutic interventions.

1.12 Rare Manifestations of Dengue: Meningitis and its Clinical Presentation

Dengue-associated meningitis, while a rare occurrence, has been documented in medical literature, offering insights into its clinical presentation and diagnostic challenges. The rarity of this condition was highlighted by Soares et al. in their study from 2006, which noted the infrequent occurrence of meningitis in dengue infections (Soares et al., 2006).

In the existing literature, there are three notable case reports of dengue meningitis. Soares et al. detailed a case involving a 24-year-old woman who presented with fever, headache, and nuchal rigidity. Diagnosis in this case was confirmed through polymerase chain reaction (PCR) and positive findings in cerebrospinal fluid (CSF) evaluations (Soares et al., 2010). Similarly, Mamdouh et al. reported two additional cases in 2013, where patients in a dengue-endemic area exhibited fever, headache, neck rigidity, and low platelet count. Diagnosis in these cases was

established based on positive IgM findings in the CSF, in the absence of the typical clinical spectrum of dengue infection. Notably, one of the cases had normal CSF cellularity, and brain CT or MRI scans were normal for both patients (Mamdouh et al., 2013). Interestingly, dengue meningitis has been reported to cause migraine-like headaches, which often show poor response to traditional anti-migraine therapies and common analgesics. The prognosis in the reported cases was positive, with patients achieving full recovery after several months without any residual neurological deficits.

These case studies emphasize the need for heightened clinical awareness and diagnostic consideration of dengue in patients presenting with meningitis-like symptoms, especially in endemic areas. The variability in clinical presentation, from typical meningitis symptoms to more subtle manifestations like resistant headaches, poses diagnostic challenges. Continued research and documentation of such cases are essential for enriching our understanding of this rare but significant manifestation of dengue infection, aiding in the development of more effective diagnostic and therapeutic approaches.

1.13 Comparative Study of Neurotropism in Dengue, Japanese Encephalitis, and Rabies Viruses

The study of neurotropic viruses, particularly Dengue virus (DENV), Japanese Encephalitis virus (JEV), and Rabies virus (RABV), reveals significant insights into their mechanisms of neuropathogenesis. These viruses, primarily prevalent in Asia, Australia, and the Western Pacific, pose considerable public health challenges due to their neuropathological disorders.

JEV, a significant RNA virus, is known to cause subclinical or mild febrile infections in most individuals. However, a small proportion of patients develop encephalitis or meningoencephalitis, often leading to fatal outcomes or permanent neurological impairments. The neuroinfection process is characterized by the disruption of the blood-brain barrier (BBB)

and extensive brain inflammation (Mishra and Basu, 2008). On the other hand, rabies, caused by RABV, remains a severe public health issue worldwide, particularly in developing countries. India, for example, is a high-risk environment due to the prevalence of canine rabies (Kumar et al., 2017). In terms of neurotropism and neuro-invasion, JEV has been demonstrated to infect various cell types present in the BBB, including brain microvascular endothelial cells (BMECs), microglia, astrocytes, and pericytes. JEV infection does not necessarily lead to cell death but can increase the permeability of the endothelial monolayer. This suggests that virus-induced cell death is not the primary mechanism for BBB disruption and viral invasion. Instead, JEV infection diminishes the expression and alters the localization of adherent and tight junction proteins (Chen et al., 2011).

Dengue, traditionally considered a non-neurotropic virus, has shown evidence of neurotropism and neuro-invasion. Although dengue fever (DF) is usually self-limiting with rare fatal outcomes, in vitro experiments have indicated that DENV can directly infect neurons, causing permanent damage. A study by Jan et al. demonstrated that mechanisms such as phospholipase A2 activation and cytochrome C release from mitochondria could lead to neuronal apoptosis in dengue infection (Jan et al., 2000). The clinical signs of DF mostly comprise endothelial cell damage and increased vascular permeability.

RABVs, in contrast, maintain the integrity of the BBB, evading immune clearance from CNS tissues (Jackson, 2002). The interaction of these viruses with BBB cells is crucial for understanding their neurotropism and neuropathogenesis. Studies on JEV and RABV have provided significant insights into how these viruses breach the BBB, leading to neurological disease. For instance, pericytes and astrocytes are both permissive to JEV replication. In vitro infection of these cells results in the release of soluble mediators that degrade tight junction proteins, particularly zonula occludens-1 (ZO-1). This effect is partially dependent on secreted proteases and inflammatory cytokines like IL-6 (Wang et al., 2012). Moreover, JEV-infected

astrocytes induce degradation of ZO-1 and claudin-5, contributing to BBB permeability. These findings indicate that JEV-induced BBB breakdown is more of a bystander event rather than a direct result of virus replication in BMECs.

Comparatively, the study of BBB interaction in DENV infection remains less clear. While DENV shows tropism mainly for monocytes, macrophages, and dendritic cells, its interaction with neuronal cells and the BBB is still under investigation. Understanding the distinctions and similarities in the mechanisms of BBB infection and disruption among these neurotropic viruses is vital for developing targeted therapies and preventive measures against these infections. This comparative study on BBB cells could greatly enhance our knowledge of the neurotropism and neuropathogenesis associated with DENV, JEV, and RABV.

Chapter 2

Review of Literature

In recent years, the world has witnessed an alarming increase in viral epidemics and pandemics, causing significant mortalities worldwide. This rising trend in viral infections has been a major public health concern, as noted in the studies by Mujtaba et al. and Irfan et al. (Mujtaba, Ghulam, et al. 2021; Irfan, Muhammad, et al. 2017). One of the most prevalent viral infections is Dengue, a mosquito-borne disease characterized by high rates of mortality and morbidity. Dengue virus has a far-reaching impact, affecting an estimated 2.5 billion people globally, as highlighted in the research by Khursheed et al. and Caraballo and King (Khursheed, Muhammad, et al. 2013; Caraballo, Hector, King, Charles., 2014). As of early 2023, the Pan American Health Organization (PAHO) reported a significant number of Dengue cases in the Americas. Nicaragua reported the highest number of cases at 4,297, closely followed by Colombia with 4,118 cases. Paraguay, Mexico, and Ecuador also reported high numbers of cases, with 3,790, 1,951, and 1,670 cases, respectively. These statistics reflect the pervasive nature of Dengue across different countries (PAHO, 2023). Moreover, there have been concerning reports of severe cases associated with the four DENVI variants (DENVI 1–4). This diversity in the viral strains indicates the evolving nature of the Dengue virus and underscores the need for ongoing vigilance and research the *Aedes aegypti* mosquito, particularly females. DENV infections can start with a mild fever but may quickly escalate to high fever and severe symptoms within a few days to two weeks post-infection (Anderson, Kathryn B., et. al., 2006; Tuiskunen Bäck, et al, 2012; Bhatt, Samir, et al. 2013; Basurko, C., et al. 2018).

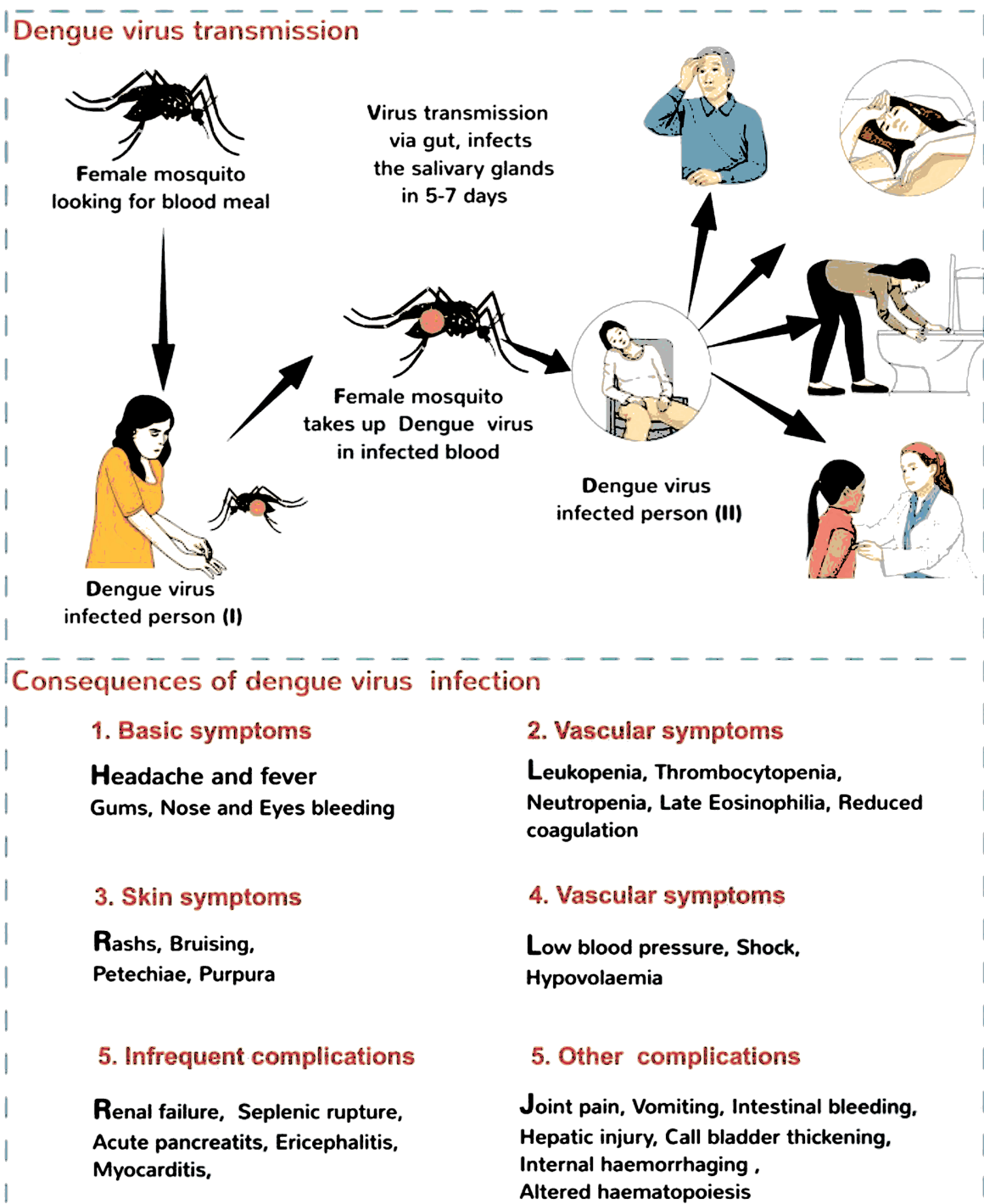


Fig. 1 Dengue virus transmission. (Source credit: Sabir, Mernan Jamal et al. “Dengue and human health: A global scenario of its occurrence, diagnosis and therapeutics.” *Saudi journal of biological sciences* vol. 28,9 (2021): 5074-5080.)

Efforts to control the spread of DENV have led to various preventive strategies. However, the presence of different DENV variants and global human travel continue to facilitate its transmission. The number of deaths attributed to DENV increased from 960 in 2000 to 4,032 in 2015, although recent times have seen a significant reduction in mortality rates. The origins of DENV in humans remain elusive, partly due to the disease's often asymptomatic nature. The first documented case of Dengue fever (DF) dates back to 1779 in Batavia, and by 1780, it was recognized as a pandemic in Philadelphia, USA (McCallum, 2008). The World Health Organization reported approximately 1.2 million DF cases in 1998 (WHO, 1998), and this number increased by 30 times over the next 50 years. An additional case was documented in China in 1992 by a medical encyclopedia. The expansion of world shipping and urbanization in port cities between the 18th and 19th centuries facilitated the spread of DENV by creating ideal environments for the primary mosquito vector, *Aedes aegypti*. The first dengue pandemic occurred simultaneously in Africa, Asia, and North America. Guzman and Istúriz report approximately 50 million DF cases in one hundred countries worldwide (Guzman, Maria G., Istúriz, R. Eduardo., 2010). DENV is transmitted between humans by infected *Aedes* mosquito species (*Ae. aegypti* or *Ae. albopictus*), as noted by Carrington and Simmons (Carrington, Lauren et al., 2014). These mosquitoes are also vectors for the West Nile virus. Gubler highlights that ecological changes in Southeast Asia have led to a dengue hemorrhagic fever (DHF) epidemic (Gubler, Duane J. 2002). The Flavivirus genus contains less than fifty species of arthropod-borne viruses, characterized by small-enveloped viruses with RNA genomes ranging from 9000 to 13,000 bases. Symptoms of DHF include plasma leakage, hypovolemia, increased vascular permeability, and shock (WHO, 1997; Gubler, 1998). In 2019, WHO reported about 4.2 million cases of the dengue virus. The growing prevalence of Dengue and its variants in worldwide necessitates a concerted effort to understand the epidemiology of the

disease better, improve clinical management, and develop effective strategies for prevention and control. The situation calls for a multidisciplinary approach, involving public health initiatives, research on vaccine development, and strategies for vector control to mitigate the impact of this global health challenge.

2.1 The Complex Role of the Blood-Brain Barrier in Pathogen-Induced Neurological Disorders

The blood-brain barrier (BBB) plays a crucial role in maintaining the central nervous system's (CNS) integrity and is an essential factor in the pathogenesis of various CNS infections that cause significant morbidity and mortality globally. Kim's research underscores the importance of the BBB as a protective barrier against pathogens, despite its vulnerability to certain infectious agents (Kim, Kyoung S., 2008). The BBB, a component of the neurovascular unit, is composed of brain endothelial cells lining cerebral microvessels. These cells are connected by tight junctions (TJs), ensuring the BBB's integrity and functioning.

This complex system involves interactions between endothelial cells and other CNS cells, including neurons, pericytes, mast cells, and glia, as described by Abbott et al. and Michiels (Abbott, N. Joan, et al. 2003). Pathogens crossing the BBB is a critical step for CNS invasion, leading to various pathologies like increased permeability, pleocytosis, and encephalopathy. Bencurova et al. elaborate on the mechanisms of BBB traversal, including paracellular, transcellular, and the “Trojan horse” mechanism, where pathogens penetrate in infected phagocytes (Bencurova, Elena, et al. 2011). These pathways often involve alterations in TJ proteins and the cytoskeleton, as detailed by Pulzova et al. (Pulzova, Lucia, et al. 2009). Various viral agents, including human immunodeficiency virus type 1, rabies virus, different flaviviruses, herpes simplex virus, and others, can cause CNS infections through BBB disruption (Spindler, Kristen R., and Hsu, 2012).

The permeability of the BBB is modulated by factors like cytokines, chemokines, matrix metalloproteinases, and others. Spindler and Hsu discuss how these factors contribute to the mechanisms employed by infectious agents to compromise the BBB, with the severity of damage varying based on the interplay of these factors (Spindler and Hsu, 2012).

Furthermore, protein-protein interactions between pathogen ligands and host receptors play a pivotal role in triggering translocation processes, particularly through paracellular and transcellular pathways (Bencurova et al., 2011). Understanding the complex dynamics of the BBB in the context of pathogen infections is crucial for developing strategies to prevent and treat neurological disorders caused by these pathogens. The multi-faceted nature of BBB crossing by pathogens highlights the need for a comprehensive approach to study the interactions at the molecular level, which can pave the way for innovative therapeutic interventions.

The mechanism of viral invasion of the central nervous system (CNS) presents complex challenges, particularly for viruses such as HIV-1, Rabies virus, and Flaviviruses. HIV-1, for instance, employs a 'Trojan horse' mechanism, entering the CNS within infected monocytes. This infiltration is facilitated by the normal turnover of perivascular macrophages or the production of pro-inflammatory mediators like MCP-1, which can compromise the blood-brain barrier (BBB) (Kim, Kyoung S. 2008). Additionally, the penetration of infected CD4⁺ T cells into the brain can be another pathway for HIV infection in the CNS (Persidsky, Yuri, and Gendelman, Howard E. 2003).

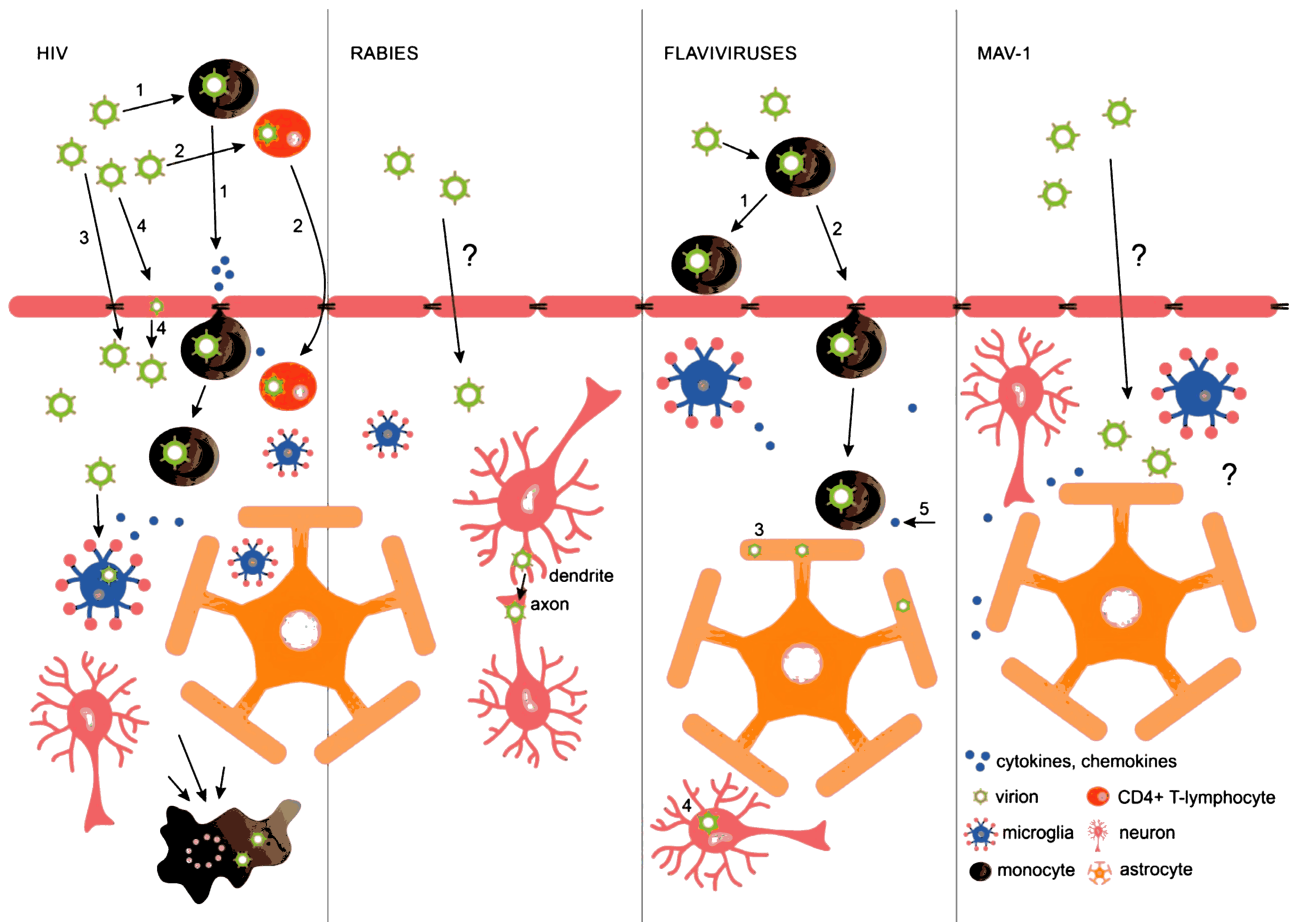


Fig 2 Routes of viral entry into CNS

The virus might also directly enter the CNS or through transcytosis of brain microvascular endothelial cells (Toborek, Michal, et al. 2001). Once the virus has penetrated the BBB, microglia can become infected and contribute to virus production. Infection of astrocytes, however, is known to be restricted (Nath, Avindra. 2002). The mechanism by which Rabies virus penetrates the BBB remains largely unknown. Following CNS infection, Rabies virions are released at synapses and use retrograde trans-synaptic pathways to infect neighboring neurons (Rupprecht, Charles E., et al. 2002). Flaviviruses, on the other hand, can infiltrate the CNS either by adhering to brain microvascular endothelial cells or via the infiltration of infected monocytes across the BBB. This results in the infection of both BBB and CNS cells. The

infection of astrocytes leads to chemokine production, facilitating further recruitment of monocytes and macrophages. Infected neurons undergo apoptosis and activate resident microglia, which produce an inflammatory response (Solomon, Tom, et al., 2004). The production of inflammatory cytokines, chemokines, enzymes, and matrix-metalloproteinases leads to the degradation of the endothelial barrier and the release of inflammatory factors, which recruit CD4⁺ and CD8⁺ T lymphocytes into the CNS parenchyma, potentially causing further inflammation and CNS damage.

Encephalitis caused by Rabies virus-1 is characterized by the recruitment of inflammatory cells, secretion of cytokines and chemokines, and alteration of tight junctions protein levels and localization in brain endothelial cells. These processes lead to neuroinflammation, neuronal damage, and BBB disruption (Anirban, et al., 2015). However, the specific mechanisms used by Rabies virus-1 to penetrate the BBB are still not well understood. Understanding these viral mechanisms is crucial for developing therapeutic strategies to prevent and mitigate the neurological damage caused by these infections.

The Rabies virus (RABV) is a member of the genus *Lyssavirus* within the *Rhabdoviridae* family, as identified by Paweska et al. It is capable of infecting almost all warm-blooded organisms and typically spreads to humans through infected saliva via animal bites or scratches. RABV continues to be a significant global health problem, causing over 70,000 human deaths annually (Wang, Haitao, et al. "Rabies and Rabies Virus." *Journal of Clinical Virology*, vol. 58, no. 4, 2013). The virus is endemic in most African and Asian countries (Nel, Louis H., et al. 2013). Clinical manifestations of rabies in humans present in two forms. The furious form, accounting for about 80% of infections, is characterized by symptoms such as hydrophobia, excitation, muscle spasms, and hallucinations. Infected animals often exhibit extreme aggression. The numb form, comprising about 20% of infections, is marked by weakness and flaccid paralysis, often leading to misdiagnosis in the early stages. In both forms, survival post-

symptom onset rarely extends beyond seven days (Schnell, Matthias J., et al. 2010). Transmission of RABV primarily occurs via saliva following a bite from an infected animal, such as dogs and cats, or through mucous membranes. It does not transmit through intact skin (Finnegan, Christopher J., et al. 2002). Other known reservoirs and transmitters include coyotes, foxes, jackals, mongooses, raccoons, skunks, wolves, and bats (Arai, Yuji. 2005). It was long believed that rabies infection in humans was invariably lethal once the virus reached the CNS. However, this notion has been challenged by findings suggesting that enhanced BBB permeability plays a critical role in survival, as it allows immune cells to enter the CNS and combat the infection (Anirban, 2013). The pathogenesis of RABV is a multigenic trait. Dietzschold et al. have shown that the RABV glycoprotein is crucial in RABV pathogenesis, controlling virus uptake and trans-synaptic spread, and regulating virus replication. Additionally, viral elements that regulate gene expression, particularly the expression of the L gene, are likely to play a role in RABV pathogenesis (Dietzschold et al., 2005). RABV binds to nicotinic acetylcholine receptors at the neuromuscular junction and travels within motor and sensory axons to the CNS. There is also spread of the virus from the CNS along neuronal pathways to infect salivary glands, skin, heart, and other organs, eventually leading to secretion into the saliva for transmission to other hosts (Jackson, Alan C. 2000). Understanding the complex mechanisms of RABV infection and its spread is essential for developing effective prevention and treatment strategies, especially given the global impact of this virus on public health.

2.2 The Emerging Global Health Challenge of Flaviviruses

The genus Flavivirus, part of the Flaviviridae family, comprises over 70 RNA viruses that include well-known human, animal, and zoonotic pathogens. This diverse group has been extensively studied by Ashraf et al., Blazquez et al., and Huhtamo et al. (Ashraf, Uzma, et al.

2015; Blázquez, Ana-Belén, et al. 2014; Huhtamo, Eili, et al., 2014). These viruses, transmitted primarily by mosquitoes or ticks, cause conditions ranging from encephalitis to systemic hemorrhagic septicemia in animals and humans (McVey et al. 2013).

Flaviviruses are found globally, with their presence documented on all continents except Antarctica, (Ashhurst et al. 2013). Factors such as globalization, climate change, changes in land use, and vector behavior have contributed to their emergence as a significant global health concern (Blázquez et al., 2014).

The mechanism by which flaviviruses breach the CNS is not fully understood, but it is believed that protein-protein interactions at the BBB play a crucial role. Turtle et al. suggest that these interactions are key to flavivirus CNS invasion (Turtle, Lance, et al. 2012). According to Neal, flaviviruses can disrupt the BBB both indirectly, through systemic inflammatory cytokines, and directly by binding to structural proteins like claudins (Neal, Jonathan, 2014).

Upon infiltration into the CNS, flaviviruses infect BBB and CNS cells, leading to a cascade of pathogenic events. Infection of astrocytes results in chemokine production, which facilitates the further recruitment of monocytes and macrophages. Infected neurons undergo apoptosis and activate resident microglia, triggering an inflammatory response. The production of inflammatory cytokines (such as $\text{TNF-}\alpha$, $\text{IL1}\beta$, $\text{INF-}\gamma$, and IL-4), chemokines (including MCP-1, CCL5, CXCL9, and CXCL10), enzymes like COX2, and matrix-metalloproteinases leads to the degradation of the endothelial barrier. This degradation allows inflammatory factors to recruit CD4^+ and CD8^+ T lymphocytes into the CNS parenchyma, potentially causing further inflammation and CNS damage (Daep, Carolyn A., et al., 2014). Understanding the complex interactions and pathogenic mechanisms of flaviviruses is critical for addressing the increasing global health challenge they pose. This knowledge is essential for developing targeted therapeutic and preventive strategies to mitigate the impact of these viruses on public health.

2.3 West Nile Virus: A Global Health Perspective and Its Pathophysiological Mechanisms

West Nile virus (WNV), a mosquito-borne flavivirus, primarily circulates in a transmission cycle between mosquito vectors and avian reservoir hosts. Aquatic birds often serve as the primary reservoirs, with infection in accidental hosts such as humans or horses usually resulting in low-level viremia and playing a minor role in the virus's life cycle (Suen, William W., et al. 2014). Human-to-human transmission of WNV is possible, albeit rare, typically occurring through blood transfusion or organ transplantation (Di Sabatino, David, et al, 2014).

Endemic to various regions of Africa, Asia, and the Middle East, WNV has become the leading cause of arboviral encephalitis in North America (Ashhurst, Thomas M., et al. 2013). It is a prominent member of the Japanese encephalitis virus complex. In humans, WNV infection is mostly asymptomatic, but it can cause mild, influenza-like symptoms. Vulnerable populations, including the elderly, chronically ill, and immunocompromised individuals, are at risk of developing severe encephalitis, which can be fatal. In horses, the infection often remains subclinical, though some animals may exhibit neurological symptoms and develop fatal encephalitis (Di Sabatino et al., 2014).

The mechanism of WNV invasion into the brain remains an area of ongoing research. WNV-associated encephalitis is marked by BBB disruption, increased immune cell infiltration into the CNS, microglial activation, inflammation, and potential neuronal loss. It is also hypothesized that WNV may enter the CNS via a transcellular pathway without compromising the BBB integrity. WNV does not typically induce a cytopathic effect but can influence the expression of claudin-1 and upregulate vascular cell adhesion molecule-1 (VCAM-1) and E-selectin (Pulzova, Lucia, et al. 2009).

The innate immune system's pattern recognition receptors detect viral RNA, triggering responses against WNV. Daniels et al. demonstrated that WNV pathogen-associated molecular patterns orchestrate endothelial responses to the virus via interactions with innate immune

cytokine signals at the BBB. While Th1 cytokines increase BBB permeability, type I interferon induced by WNV promotes and stabilizes its function. The induction of innate cytokines via pattern recognition pathways directly regulates BBB permeability and tight junction formation through the balanced activation of small GTPases Rac1 and RhoA. This regulation impacts the trans-endothelial trafficking of the virus. In vivo experiments on mice with attenuated type I interferon signaling or interferon induction (Ifnar (-/-) Irf7(-/-)) exhibited enhanced BBB permeability and tight junction dysregulation following WNV infection (Daniels, Brian P., et al. 2014). Understanding the pathophysiological mechanisms of WNV infection and its impact on the BBB is crucial for developing effective prevention and treatment strategies, particularly given the virus's global prevalence and potential for severe neurological complications.

2.4 Understanding the Pathophysiology of Tick-Borne Encephalitis Virus (TBEV)

Tick-borne encephalitis virus (TBEV) is a significant cause of severe encephalitis in humans, often leading to serious sequelae. Unique among flaviviruses, TBEV can be transmitted through the consumption of non-pasteurized milk from infected cows and goats, although ticks remain the primary vector for virus transmission. Ixodes ticks, especially *I. scapularis*, *I. ricinus*, and *I. persulcatus*, prevalent in Central and Eastern Europe, are the primary vectors for TBEV (Ruzek, Daniel, et al. 2011).

The exact mechanisms by which TBEV accesses the CNS remain to be fully elucidated. Several hypothetical routes for TBEV traversal across the blood-brain barrier (BBB) have been proposed, including cytokine-mediated BBB breakdown, the “Trojan horse” theory involving infected immune cells, and direct viral entry into brain microvascular endothelial cells followed by transcytosis and release into the brain parenchyma (Ruzek et al., 2011).

Tick-borne encephalitis is typically characterized by neurological disorders, but other symptoms such as mild fever and itching may also occur. Approximately 20% of infected

patients risk developing chronic or permanent neuropsychiatric sequelae (Kaiser, Reinhard. 2008). The virus can infect both the meninges and the brain. Recent advances in electron tomography have shed light on the molecular events of TBEV infection in neurons, suggesting similar processes in brain microvascular endothelial cells. Bily et al. observed direct connections between tubule-like structures in neurons and viral particles in the endoplasmic reticulum, with viral particles also found in cellular microtubules and vacuoles (Bily, Tomas, et al. 2015). Aside from neurons, TBEV also infects astrocytes, which are situated between synapses and endothelial cells. Astrocytes play several roles in the brain, including neuronal support and, importantly, the maintenance of tight junctions under both normal and pathological conditions (Tao-Cheng, Jung-Hwa, et al. "The Role of Astroglia in Blood-Brain Barrier Formation and Function." *Journal of Electron Microscopy Technique*, vol. 7, no. 3, 1987). It has been shown that TBEV infection alters the permeability of the BBB, with astrocytes potentially implicated in this process due to their role in regulating brain blood flow (Potokar et al., 2014). This growing body of research on TBEV's pathophysiology enhances our understanding of its impact on the CNS, paving the way for more effective prevention and treatment strategies for this debilitating condition.

2.5 Japanese Encephalitis Virus: Epidemiology, Pathogenesis, and Neurological Impact

Japanese encephalitis virus (JEV) is part of a group that includes three other significant viruses: Saint Louis encephalitis virus, Murray Valley encephalitis virus, and West Nile virus. As an etiological agent causing Japanese encephalitis, JEV's impact on public health is considerable (Murphy, Frederick A. 1999). While JEV infection is often inapparent, it can lead to clinical disease in humans, horses, and swine (McVey, D. Scott, et al. 2013).

Endemic in Southeastern and Central Asia, JEV results in approximately 30,000–50,000 cases annually. The spectrum of symptoms in humans ranges from mild febrile illness to severe

encephalitis, including seizures, polio-like illness, and various movement disorders (Ashhurst, Thomas M., et al., 2013). Fatal cases often exhibit pathological changes like vascular congestion, cerebral edema, neuronal death, astrocyte activation, and microglial proliferation in the nervous system.

JEV transmission between animal and human hosts primarily occurs through *Culex* species mosquitoes. Despite its significance, details about the pathogenesis of human JEV infection, including its spread to the CNS and viral tropism within the brain, remain limited. In vitro studies have shown that peripheral blood mononuclear cells, including monocytes and macrophages, can be infected and invade the CNS via anterograde transport of virions or through vascular endothelial cells. This suggests that JEV may undergo a peripheral replication cycle. Many flaviviruses induce neuronal apoptosis in neurons in vitro and in rodent models, a hallmark of neurodegenerative infections. JEV, specifically, can cause neuronal loss via the rough endoplasmic reticulum stress pathway. Viral tropism in neural progenitor stem cells and immature neurons has been observed, although mature neurons exhibit resistance to JEV-induced apoptosis due to increased expression of cellular inhibitors of apoptosis such as Bcl-2 and Bcl-x.

In addition to neurons, astrocytes and microglial cells can also be infected. Recent studies in human and mouse models have shown prominent astrocyte activation, especially in areas of neuronal damage. Ghoshal et al. (2007) reported a significant increase in various proinflammatory mediators, including inducible nitric oxide synthase, cyclooxygenase 2, interleukin 6, interleukin 1b, tumor necrosis factor-alpha, and monocyte chemoattractant protein 1, in microglial cells following JEV infection, which may play a critical role in inducing neuronal cell death (Zhang, Shuxian, et al. 2014). The understanding of JEV's neurotropism and pathogenesis is essential for developing effective prevention and treatment strategies, particularly considering the virus's significant impact on human health in endemic regions.

2.6 Rabies Virus: Pathogenesis and Blood-Brain Barrier Interactions

Rabies virus (RABV), a negative-stranded RNA virus of the genus *Lyssavirus* within the *Rhabdoviridae* family, is a significant public health concern, causing fatal encephalomyelitis and resulting in over 55,000 human deaths annually. Knobel et al. highlight RABV's global impact, particularly in regions where vaccination and control measures are limited (Knobel, Darryn L., et al. 2005). RABV typically enters the body through a wound site and progresses to the central nervous system (CNS) via sensory and motor neurons. Despite its severe clinical symptoms, RABV infection often results in surprisingly little tissue damage or neuronal pathology in the brains of affected patients, as observed by Shankar et al. (Shankar, Vijay, et al. 2011).

Studies in mouse models have shown that wild-type (wt) RABV infection typically causes mild inflammation. In contrast, laboratory-attenuated RABV strains lead to more extensive inflammation, apoptosis, and expression of innate immune genes in the CNS, as discussed by Jackson et al. (Jackson, Alan C., et al. 2008). Dietzschold et al. found that BBB permeability is notably enhanced in mice infected with laboratory-attenuated, but not wt, RABV (Dietzschold, Bernhard, et al. 2001). This increased BBB permeability is significant in RABV attenuation, as it allows immune effectors to enter the CNS and eliminate the virus, as noted by Hooper et al. (Hooper, D. Craig, et al. 1998).

The BBB, composed of endothelial cells, pericytes, and astrocytes, acts as a barrier against the invasion of the CNS by macromolecules and microorganisms. Alteration of BBB permeability is a common occurrence in both bacterial and viral infections, with tight junction (TJ) damage in brain microvascular endothelial cells (BMECs) being a key mechanism of BBB breakdown. The TJ complex, consisting of transmembrane TJ proteins (occludin and claudins) and cytosolic

TJ proteins (zonula occludens-1 [ZO-1]), is crucial for maintaining BBB integrity, as highlighted by Lehner et al. (Lehner, Michael D., et al. 2011).

Several viral infections, such as those caused by HIV, JEV, and mouse adenovirus type 1 (MAV-1), can alter BBB permeability, (Toborek et al. 2016). For instance, MAV-1 disrupts the TJ complex directly in primary ECs (Gralinski, Lisa E., et al. 2009), while HIV enhances BBB permeability via the induction of chemokines like CCL2 in the CNS (Toborek et al., 2016). This review summarizes current knowledge on how various viruses, invade the CNS. It illustrates the diverse mechanisms by which viruses can cross the BBB in a wide array of infectious diseases, underscoring the complexity of viral pathogenesis and the importance of understanding these mechanisms for the development of effective therapeutic.

Aim:

Our aim to see the effect of Dengue, Japanese Encephalitis virus and Rabies virus when exposed to blood-brain barrier endothelial cells.

Objectives:

- 1) To evaluate the neurotoxicity of DENV, JEV, RABV viruses on cerebral microvascular endothelial cells
- 2) To study effect of inflammatory cytokine on DENV, JEV, RABV virus infection on endothelial cells
- 3) To study the effect of DENV, JEV, RABV virus on endothelial cells tight junction protein
- 4) To evaluate the effect of DENV, JEV, RABV virus infection on MMP-2 and MMP-9 matrix metalloproteinases

Hypothesis:

Exposure of blood-brain barrier endothelial cells to *flaviviruses* such as Japanese Encephalitis virus does elicit distinct neurotoxic responses, inflammatory cytokine effects, perturb tight junction proteins. The study hypothesizes that Dengue virus exposure might disrupt the blood-brain barrier (BBB) integrity, influencing BBB permeability and ultimately leading to neurological damage. This comparative investigation aims to enhance our understanding of BBB infections by neurotropic viruses such as DENV, JEV, and RABV, shedding light on neurotropism and neuropathogenesis associated with DENV.

Chapter 3

Materials and Method

Materials and Methods

3.1 In silico studies of dengue envelope protein

The objective of this experiment is to investigate the interaction between the dengue envelope protein (DEP), the outermost coating of the dengue virus, and blood-brain barrier endothelial cell receptors. This study aims to elucidate whether DEP plays a role in viral entry into the brain, which has significant implications for understanding the pathogenesis of dengue virus infection. The significance of this experiment lies in its potential to shed light on the mechanisms of dengue virus neuroinvasion, a critical aspect of the disease's progression. By employing a series of computational techniques, including homology modeling, docking, and energy minimization, this research seeks to provide insights into the molecular interactions between DEP and the transmembrane receptors, ultimately contributing to our understanding of dengue virus neuropathogenesis and potentially facilitating the development of targeted therapeutic interventions.

1. Sequence Retrieval: Retrieve the amino acid sequence of DEP (AAC59274.1, Phe247-Gly675) from Uniprot.
2. Homology Modeling: Construct the 3D structure of Dengue envelope protein (DEP) using the SWISS-MODEL homology modeling server. Utilize BLAST with 98.73% sequence similarity as a template for modeling.
3. Structure Preparation: Obtain 3D structures of the brain endothelial transmembrane receptor from Uniprot and Protein Data Bank (PDB) databases and remove heteroatoms from the receptor structures for protein refinement.
4. Docking Preparation: Perform protein-protein docking between DEP and the membrane receptor protein using the PatchDock program server.

5. Ranking Solutions: Rank the top 20 docking solutions based on geometric shape complementarity score.
6. Solution Optimization: Optimize the 10 best solutions from PatchDock using FireDock to improve their binding conformations.
7. Binding Energy Selection: Choose the docking solution with the lowest binding energy for further interaction analysis.
8. Energy Minimization: Obtain a collection of transmembrane proteins from a library and subject the transmembrane protein collection to energy minimization.
9. Final Docking: Perform docking between Dengue envelope protein and the energy-minimized transmembrane protein collection.
10. Binding Energy Assessment: Identify the structure with the lowest binding energy using Poisson-Boltzmann with nonpolar surface area calculations.
11. Interaction Analysis: Conduct an analysis of interactions, including hydrogen bonding and salt bridges between DEP and the transmembrane receptor.
12. Scoring: Calculate protein scores to assess the quality of the protein-protein interaction.
13. Visualization and Data Retrieval: View and download the final protein docked structure in Discovery Studio for further analysis and reporting.

(A) *In-vitro* studies of viruses on blood-brain barrier

3.2 Cell Culture

3.2.1 Murine brain endothelial cell line bEnd3 cells

These were procured from ATCC (American Type Culture Collection), USA by following an extensive procedure. bEnd3 cell lines are derived from BALB/c mice strain having endothelial morphology. The bEnd3 endothelial cell line serves as a valuable model in our research on understanding how viruses disrupt the blood-brain barrier, a critical event linked to neuropathogenesis. By utilizing these cells, we aim to investigate the mechanisms underlying viral-induced barrier dysfunction and its implications in neuroinflammatory processes, shedding light on potential therapeutic interventions for viral-associated neurological disorders. These cells were used for virus neuro-pathogenesis study.

1. Gently thaw the vial of Murine brain endothelial cell line (bEnd3 cells) by placing it in a 37°C water bath for 1-2 minutes.
2. Remove the vial from the water bath once the contents are thawed and decontaminate it by spraying 70% ethanol.
3. Transfer the vial contents to a 15ml centrifuge tube containing 9.0 mL of complete DMEM (ATCC 30-2002).
4. Centrifuge the cell suspension at 125g for 10 minutes.
5. Resuspend the cell pellet with 9ml of complete DMEM.
6. Transfer the resuspended cells into a T-25 flask.
7. Incubate the cells at 37°C in a 5% CO₂ incubator.
8. For passaging of cells, remove and discard the culture medium.
9. Briefly rinse the cell layer with 0.25% (w/v) Trypsin 0.5mM EDTA solution.

10. Add 2 ml of Trypsin-EDTA solution to the flask and observe cells under an inverted microscope until the cell layer is detached.
11. Collect the detached cells in a 15 ml tube and centrifuge at 125g for 10 minutes.
12. After centrifugation, discard the supernatant.
13. Add 10 ml of complete DMEM and aspirate cells by gently pipetting.
14. Transfer the cell suspension into a new flask, tighten the cap, and place it in the CO₂ incubator at 37°C.

3.2.2 Baby Hamster Kidney cell line (BHK-21 cells)

The BHK21 cell line is employed as a valuable tool for virus propagation in studies focused on investigating the mechanisms by which viruses disrupt the blood-brain barrier and induce neuropathogenesis. By infecting BHK21 cells with the target virus, we can simulate the initial stages of infection and study the virus's interactions with cellular components relevant to the blood-brain barrier. This model provides insights into the virus's ability to cross this critical barrier and potentially lead to neurological complications, shedding light on the underlying pathogenic processes.

1. For cell revival which were stored in liquid nitrogen, thaw the frozen BHK-21 cell line vials by placing them in a water bath.
2. Prepare 5 ml of MEM medium with 20% FBS in a 15 ml Sterile Centrifuge Tube (1 ml FBS + 4 ml MEM + 125 µL HEPES).
3. Transfer the contents of the thawed vial into the centrifuge tube.
4. Centrifuge the tube at 1500 rpm at 20-25°C for 10 minutes.
5. Discard the supernatant and resuspend the cell pellet in 5 ml of Complete MEM medium (with 20% FBS).
6. Centrifuge the tube again at 1500 rpm at 20-25°C for 10 minutes.

7. Meanwhile, prepare 10 ml of Complete MEM with 20% FBS and 250 μ L HEPES in a sterile T-25 Flask.
8. Discard the supernatant from the centrifuge tube and add 2 ml of Complete MEM from the T-25 Flask into the centrifuge tube with the cell pellet. Gently resuspend the pellet using the pipetting method.
10. Transfer the contents of the centrifuge tube into the T-25 Flask and gently swirl the flask to distribute the cells evenly.
11. Incubate the flask at 37°C with 5% CO₂ in a CO₂ incubator until a confluent monolayer is achieved. Remove and discard the culture medium. Briefly rinse the cell layer with 0.25% (w/v) Trypsin 0.5mM EDTA solution.
14. Add 2 ml of Trypsin-EDTA solution to the flask and place it in the CO₂ incubator to facilitate trypsinization at optimal temperature conditions.
15. Observe the cells under an inverted microscope until the cell layer is detached.
16. Collect the detached cells in a 15 ml tube and centrifuge at 1500 rpm for 7 minutes.
17. After centrifugation, discard the supernatant and loosen the cell pellet by gently flicking the tube. Add 5 ml of complete MEM and aspirate the cells gently by pipetting.
19. Transfer the cell suspensions into a new flask, tighten the cap, and place it in the CO₂ incubator at 37°C.

3.2.3 Neuronal cell line (N2a cells)

The N2a neuronal cell line is a valuable tool in studying viruses that disrupt the blood-brain barrier and induce neuropathogenesis. By infecting N2a cells with the virus of interest, we can investigate the mechanisms underlying the virus's ability to breach the blood-brain barrier and cause neurological damage. This cell line serves as an in vitro model to better understand the

pathogenesis of neurotropic viruses and develop potential therapeutic interventions to mitigate their impact on the central nervous system.

1. Revive the frozen N2a neuronal cell line from liquid nitrogen storage by thawing the vials in a water bath.
2. Prepare a 15 ml Sterile Centrifuge Tube with 5 ml of MEM medium containing 20% FBS (1 ml FBS + 4 ml MEM + 125 μ l HEPES).
3. Transfer the content of the thawed vial into the prepared centrifuge tube.
4. Centrifuge the tube at 1500 rpm at 20-25°C for 10 minutes.
5. Discard the supernatant and resuspend the cell pellet in 5 ml of Complete MEM medium (with 20% FBS and 250 μ l HEPES).
6. Centrifuge the tube again at 1500 rpm at 20-25°C for 10 minutes.
7. Prepare 10 ml of Complete MEM with 20% FBS in a Sterile T-25 Flask.
8. Discard the supernatant from the centrifuged tube and add 2 ml of Complete MEM from the T-25 Flask to the centrifuge tube with the cell pellet.
9. Gently resuspend the pellet using pipetting.
10. Transfer the contents of the centrifuge tube into the T-25 Flask and gently swirl the flask to distribute the cells throughout.
11. Incubate the Flask at 37°C with 5% CO₂ in a CO₂ incubator until a confluent monolayer is achieved.
12. For cell passaging remove and discard the old culture medium and briefly rinse the cell layer with 0.25% (w/v) Trypsin 0.5 mM EDTA solution.
13. Add 2 ml of Trypsin-EDTA solution to the flask.
14. Observe cells under an inverted microscope until the cell layer is detached.
15. Collect the detached cells in a 15 ml tube and centrifuge it at 1500 rpm for 7 minutes.
16. Discard the supernatant and loosen the pellet by gently flicking.

17. Add 5 ml of complete MEM and aspirate cells by gently pipetting.
18. Transfer the cell suspensions to a new flask, tighten the cap, and keep it in the CO₂ incubator at 37°C and use it for further experiments.

3.2.4 Neuronal cell line (SH-SY-5Y cells)

The SH-SY5Y neuronal cell line serves as a valuable in vitro model for studying viruses that disrupt the blood-brain barrier and induce neuropathogenesis. By infecting these cells with relevant neurotropic viruses, researchers can investigate the mechanisms by which viruses breach the blood-brain barrier and interact with neural cells, providing insights into potential therapeutic targets to mitigate virus-induced neuroinflammatory responses and neurologic diseases.

1. Thaw the frozen neuronal cell line (SH-SY-5Y cells) by placing the vials in a water bath.
2. Prepare a 15 ml Sterile Centrifuge Tube and add the following components to make sterile revival media (5 ml MEM medium + 20% FBS (1 ml FBS) + 4 ml MEM + 125 µL HEPES + 1 mM sodium pyruvate + 0.1 mM nonessential amino acids)
3. Transfer the content of the thawed vial into the prepared centrifuge tube.
4. Centrifuge the tube at 1500 rpm at 20-25°C for 10 minutes.
5. Discard the supernatant and resuspend the pellet in 5 ml of Complete MEM medium (with 20% FBS and 250 µL HEPES).
6. Centrifuge the tube again at 1500 rpm at 20-25°C for 10 minutes.
7. Prepare a Sterile T-25 Flask containing 10 ml of Complete MEM with 20% FBS.
8. Discard the supernatant from the centrifuged tube and add 2 ml of Complete MEM from the T-25 Flask into the centrifuge tube with the cell pellet.
9. Gently resuspend the pellet by pipetting.

10. Transfer the contents of the centrifuge tube into the T-25 Flask and gently swirl the flask to distribute the cells evenly.
11. Incubate the Flask at 37°C with 5% CO₂ in a CO₂ incubator until a confluent monolayer is achieved.
12. When a confluent monolayer is achieved, passage the cells by removing and discard the old culture medium.
13. Rinse the cell layer with 0.25% (w/v) Trypsin 0.5mM EDTA solution.
14. Add 2 ml of Trypsin-EDTA solution to the flask.
15. Observe the cells under an inverted microscope until the cell layer is detached.
16. Collect the detached cells in a 15 ml tube and centrifuge the tube at 1500 rpm for 7 minutes.
17. Discard the supernatant and loosen the pellet by gently flicking.
18. Add 5 ml of Complete MEM and aspirate cells by gently pipetting. Transfer the cell suspensions into a new flask, tighten the cap, and place it in the CO₂ incubator at 37°C.

3.2.5 Murine macrophage cell line (RAW cells)

The use of RAW cell lines in studying viruses disrupting the blood-brain barrier is significant as they represent a readily available and well-characterized model of murine macrophages. Since the blood-brain barrier plays a crucial role in protecting the central nervous system, studying virus interactions with RAW cells can provide insights into the mechanisms by which viruses may compromise this barrier, potentially aiding in the development of strategies to combat neuroinvasive infections. Follow the below protocol for revival and passaging the RAW cell line.

1. Thaw the frozen cell line by placing the vials in a water bath.
2. Prepare a 15 ml Sterile Centrifuge Tube and add the components to make a sterile revival media (5 ml MEM medium + 20% FBS (1 ml FBS) + 4 ml DMEM + 4mM L-Glutamine)

3. Transfer the content of the thawed vial into the prepared centrifuge tube.
4. Centrifuge the tube at 1500 rpm at 20-25°C for 10 minutes.
5. Discard the supernatant, and resuspend the pellet in 5 ml of Complete MEM medium.
6. Centrifuge the tube again at 1500 rpm at 20-25°C for 10 minutes.
7. Prepare a Sterile T-25 Flask containing 10 ml of Complete MEM with 20% FBS and 4mM L-Glutamine.
8. Discard the supernatant from the centrifuged tube and add 2 ml of Complete MEM from the T-25 Flask into the centrifuge tube with the cell pellet.
9. Gently resuspend the pellet by using the pipetting method.
10. Transfer the contents of the centrifuge tube into the T-25 Flask and gently swirl the flask to distribute the cells evenly.
11. Incubate the flask at 37°C with 5% CO₂ in a CO₂ incubator until a confluent monolayer is achieved.
12. Remove and discard the culture medium.
13. Briefly rinse the cell layer with 0.25% (w/v) Trypsin 0.5mM EDTA solution.
14. Add 2 ml of Trypsin-EDTA solution to the flask.
15. Observe the cells under an inverted microscope until the cell layer is detached.
16. Collect the detached cells in a 15 ml tube.
17. Centrifuge the tube at 1500 rpm for 7 minutes.
18. After centrifugation, discard the supernatant, and gently flick to loosen the pellet.
19. Add 5 ml of complete MEM and aspirate cells by gently pipetting.
20. Transfer the cell suspensions into a new flask, tighten the cap, and place it in the CO₂ incubator at 37°C.

3.2.6 Porcine stable cell line (PS cells)

To investigate the disruption of the blood-brain barrier and neuropathogenesis caused by Japanese Encephalitis Virus (JEV), we employed PS porcine stable cell lines as a valuable model system for JEV propagation. These cell lines proved essential in understanding the virus's interactions with host cells and its potential role in altering the blood-brain barrier, shedding light on the mechanisms underlying JEV-induced neuropathogenesis.

1. Thaw the frozen cell line by placing the vials in a water bath.
2. Prepare a 15 ml Sterile Centrifuge Tube and add the following components to it (5 ml MEM medium + 20% FBS - 1 ml FBS + 4 ml MEM)
3. Transfer the contents of the thawed vial into the centrifuge tube.
4. Centrifuge the tube at 1500 rpm at 20-25°C for 10 minutes.
5. Discard the supernatant and resuspend the pellet in 5 ml of Complete MEM medium (with 20% FBS).
6. Centrifuge the tube again at 1500 rpm at 20-25°C for 10 minutes.
7. Prepare 10 ml of Complete MEM with 20% FBS in a Sterile T-25 Flask.
8. Discard the supernatant from the centrifuged tube and add 2 ml of Complete MEM from the T-25 Flask into the centrifuge tube with the cell pellet.
9. Gently resuspend the pellet using the pipetting method.
10. Transfer the contents of the centrifuge tube into the T-25 Flask and gently swirl the flask to distribute the cells evenly.
11. Incubate the flask at 37°C with 5% CO₂ in a CO₂ incubator until a confluent monolayer is achieved.
12. Remove and discard the culture medium from the flask.
13. Briefly rinse the cell layer with 0.25% (w/v) Trypsin 0.5mM EDTA solution.
14. Add 2 ml of Trypsin-EDTA solution to the flask.

15. Observe the cells under an inverted microscope until the cell layer is detached.
16. Collect the detached cells in a 15 ml tube.
17. Centrifuge the tube at 1500 rpm for 7 minutes.
18. Discard the supernatant and loosen the pellet by gently flicking.
19. Add 5 ml of complete MEM and aspirate the cells by gently pipetting.
20. Transfer the cell suspensions into a new flask, tighten the cap, and place it in the CO₂ incubator at 37°C.

3.2.7 BV2 cell line

The BV2 cells, as a model of microglia, are instrumental in studying BBB disruption caused by exposure to Dengue, Japanese Encephalitis Virus, and Rabies virus. This approach enables the investigation of neuroinflammatory responses and BBB integrity alterations in the context of these neurotropic viral infections.

1. Thaw frozen BV2 cells rapidly in warm water to minimize ice crystal formation.
2. Slowly dilute the cell suspension after thawing for maximum viability. Gradually dilute DMSO to avoid osmotic damage.
3. Transfer cryovial contents to a centrifuge tube with DMEM (2mM L-glutamine + 3.7 g/L Sodium Bicarbonate + 10% antibiotic-antimycotic solution) and 20% FBS, then centrifuge at 1,500 rpm for 5 minutes. Discard supernatant and add fresh DMEM and 20% FBS.
4. Transfer cells to a T25 flask and incubate overnight; check for cell adherence under an inverted microscope. Passage cells at a split ratio greater than 1:2, indicating culture age and passage number. Inspect the BV2 cell line for monolayer growth, cell degeneration, pH, and contamination. Discard contaminated or damaged flasks.

5. In a bio-safety cabinet level 2 (BSC-2), warm DMEM and FBS to room temperature for 30 minutes, activate UV light for 20 minutes, and clean the area with 70% alcohol. Select T25 flasks with complete monolayer, decant DMEM, and use a cell scraper to detach cells.
6. Resuspend cells in 5 ml DMEM with 10% FBS, distribute according to split ratio, add 0.1 ml Gentamicin, and label flasks. Incubate flasks at 37°C with 5% CO₂, subculture every 2-3 days, and examine microscopically within 24 hours.

3.2.8 C6/36 cell line

The C636 cell line serves as a valuable tool in our research to study dengue virus propagation and its role in disrupting the blood-brain barrier, leading to neuropathogenesis. By infecting C636 cells with dengue virus strains, we can investigate the mechanisms underlying viral entry, replication, and the subsequent impact on barrier integrity, providing insights into the development of potential therapeutic interventions for dengue-associated neurological complications.

1. Thaw frozen cells rapidly by placing the cryovial in a warm water/water bath to minimize intracellular ice crystal formation.
2. Dilute the cell suspension slowly after thawing to optimize recovery and ensure maximum viability. Note that gradual dilution is crucial when dealing with DMSO, as sudden dilution can cause severe osmotic damage and reduce cell survival by half.
3. Remove the frozen cryovial containing C636 cells from the liquid nitrogen canister. Thaw the cryovial in warm water. Transfer the contents of the cryovial into a centrifuge tube.
4. Add MEM (2mM L-glutamine + 3.7 g/L Sodium Bicarbonate + 10% antibiotic-antimycotic solution) with 20% FBS to the centrifuge tube. Centrifuge at 1,500 rpm for 5 minutes.

5. Discard the supernatant and replace it with MEM containing 20% FBS. Transfer the cells into a T25 tissue culture flask. Incubate overnight and observe the T25 flask for cell adherence under an inverted microscope.
6. Observe the C636 cell line in the T25 flask under an inverted microscope for complete monolayer/cell growth, cell degeneration, pH levels and microbial contamination
7. Discard contaminated or sheet-torn bottles/flasks. Handle cell lines in a biosafety cabinet level 2 (BSC-2). Allow MEM and FBS to reach room temperature (RT, 22°C-28°C) for 30 minutes.
8. Before initiating, switch on the UV light of the biosafety cabinet for 20 minutes and clean the working area of the laminar flow hood with 70% alcohol.
9. Select T25 flasks with a complete monolayer for further passaging. Decant MEM from the tissue culture flask containing C636 cell line. Harvest the cells using a cell scraper.
10. Re-suspend the cells in 5 ml of MEM containing 10% FBS. Distribute the cells into flasks based on the split ratio (1:2 – 1:3). Add 0.1 ml of Gentamicin (10 mg/ml) to each flask.
11. Label each flask with the cell name, passage number, date of passage, and initials of the person preparing the culture. Incubate the flasks at room temperature and sub-culture every 3-4 days based on the confluence of the monolayer. Examine the flasks microscopically within 24 hours.

3.3 Dengue virus Propagation

The objective of the dengue virus propagation experiment is to isolate and propagate the dengue virus using the C636 cell line. This process involves carefully following a series of steps, including inoculation, incubation, and harvesting, to produce virus stocks that can be stored for further research and analysis. The significance of this experiment lies in its role in providing researchers with a renewable and controlled source of dengue virus for various studies,

including investigations into the virus's interaction with host cells, pathogenesis, and potential vaccine or antiviral drug development. The methodology outlined in the provided reference serves as a standardized and reproducible protocol for researchers working with dengue virus, contributing to the advancement of our understanding of this important viral pathogen. By focusing on more predominant strain DENV-2, we can explore how variations in viral strains may influence the disruption of tight junction proteins and the severity of neurological complications, contributing to a more comprehensive understanding of dengue pathogenesis. Given the global prevalence of DENV-2, insights gained from this study can have significant epidemiological implications. Understanding how DENV-2 interacts with brain endothelial cells and disrupts tight junction proteins can inform public health strategies for regions where this serotype is predominant.

1. Collect the Dengue virus 2 strain stored in aliquots in cryovials from a -80°C deep freezer. Process the strain on the C636 cell line for Dengue virus isolation. Observe the C636 cell lines in T25 flasks under an inverted microscope for complete monolayer/cell growth and check for microbial contamination.
2. Select T25 flasks with a complete monolayer and no microbial contamination for isolation. Discard the medium containing a monolayer of C636 cell line. Rinse the cell sheet and decant the excess liquid. Add 500 microliters of the sample gently to the cell sheet.
3. Inoculate a control T25 flask containing the C636 cell line sheet with 0.5 ml of MEM medium. Allow the inoculation to absorb for 1 hour at 37°C with intermittent shaking for virus adsorption. After adsorption, add 4.5 ml of MEM medium to each T25 flask.
4. Incubate the inoculated flasks at room temperature. Monitor the C636 cells under an inverted microscope for ballooning for up to 7 days. Harvest tissue culture fluids typically on the 5th to 7th day of infection.

5. Centrifuge the infected cell culture supernatant at 1,500 rpm for 10 minutes to remove excess cells. Collect the supernatant fluid and aliquot it in cryovials along with glycerol as a stabilizer to a final concentration of 0.5%. Aliquot virus stocks and store them at -80°C until use.

3.4 Japanese Encephalitis Virus Propagation

The Japanese Encephalitis Virus (JEV) GP-78 strain, also known as the Japanese encephalitis virus Gorakhpur-78 strain, is a specific variant or isolate of the Japanese Encephalitis Virus. Japanese encephalitis is a flavivirus infection that is primarily transmitted by mosquitoes, particularly *Culex* species. This virus can cause Japanese encephalitis, a serious neurological disease that affects the brain. JEV GP-78 strain is neuroinvasive in nature has the ability to enter and infect the central nervous system, particularly the brain and spinal cord. Neuroinvasive strains of the virus have a higher propensity to cause neurological symptoms and complications, including encephalitis. These complications can range from mild symptoms such as headaches and fever to more severe conditions, including paralysis, seizures, and cognitive impairments.

Understanding the neuroinvasive nature of this specific strain, JEV GP-78, is essential for research and public health efforts because it helps scientists and healthcare professionals better comprehend the mechanisms of viral entry into the central nervous system. This knowledge can inform strategies for diagnosis, treatment, and prevention of Japanese encephalitis, particularly in regions where the disease is endemic. Additionally, it underscores the importance of mosquito control measures and vaccination programs to reduce the incidence of this potentially devastating disease. The propagation and titration of the JEV (GP78 STRAIN) was performed using a plaque assay in Porcine Stable (PS) cells.

1. Seed PS cells in 12-well tissue culture plates at a density of 0.2×10^5 cells per well.
2. Infect the PS cells with serially diluted cell culture-grown JEV (GP78) virus for 1 hour.

3. Remove excess virus by rinsing the cells twice with 1 X phosphate-buffered saline (PBS).
4. Prepare a 2% low-melting agarose/growth media mixture (1:1).
5. Add the agarose overlay mixture to each well and allow it to solidify at room temperature (RT) for 15 minutes.
6. Allow the cells to grow for 3 days at 37°C with 5% CO₂.
7. Fix the cells with 4% formaldehyde at 37°C for 1 hour.
8. Stain the cells by adding 1.5% crystal violet solution for 15 minutes at 37°C.
9. Wash the stained cells thrice with distilled water as described above.
10. Count the plaques to determine the concentration of virus in plaque-forming units (PFU) per mL.
11. Calculate the PFU value using the formula $PFU = N \cdot DF / V$, where N represents the number of plaques counted, DF represents the virus dilution factor, and V represents the volume of the inoculum.

3.5 Rabies Virus Propagation

The Rabies Virus CVS-11 strain, like other strains of the rabies virus, enters the central nervous system (CNS) primarily through a process known as retrograde axonal transport. This process begins at the site of the initial infection, typically a peripheral site like a muscle or skin, where the virus gains entry via a bite from an infected animal. The virus then enters peripheral nerves and utilizes the neuronal transport system to travel along nerve fibers towards the CNS, ultimately reaching the spinal cord and brain. This retrograde axonal transport allows the virus to bypass the blood-brain barrier and gain access to the central nervous system (Sánchez-Martínez, and Juan C. de la Torre).

1. Retrieve the Challenge Virus Standard Strain (CVS)-11 of rabies virus from laboratory storage.

2. Propagate the virus in BHK-21 cells.
3. Grow BHK-21 cell monolayers in T75 flasks to 90% confluence.
4. Infect BHK cells with CVS-11 at a Multiplicity of Infection (MOI) of 0.8.
5. Harvest infected cells after 72 hours of infection.
6. Perform a total of three freeze-thaw cycles to disrupt cells.
7. Centrifuge the disrupted cells to collect virions.
8. Conduct the 50% Tissue Culture Infectious Dose (TCID₅₀) assay on BHK-21 cells.
9. Seed BHK-21 cells in 96-well plates and allow them to grow overnight.
10. Infect cells with serially diluted virus samples.
11. Incubate infected cells for 5 to 7 days.
12. Examine plates under a microscope for cytopathic effects (CPE).
13. Calculate TCID₅₀ value using the Karber method formula.

3.6 Plaque Assay for Virus Titre Determination

The Plaque Assay for Virus Titre Determination is pivotal in virology, enabling precise measurement of virus concentration. It plays a critical role in vaccine development, antiviral drug testing, and characterizing viruses. Additionally, it serves as a diagnostic tool in clinical settings and aids research into viral pathogenesis, contributing to our understanding of viruses and their impact on health and disease.

1. Seed BHK-21 cells in 12-well plates to form a confluent monolayer overnight at 37 °C in a 5% CO₂ incubator.
2. Inoculate the cell monolayer with an appropriate dilution of viral culture supernatant to ensure that the final plaque counts were reliable. Incubate for 90 min at 37 °C with occasional shaking.

3. After adsorption, remove the unbound virus, and wash the cells with PBS.
4. Overlay the cells with 1 mL solid culture medium (2% FBS, 1% Aquacide II, DMEM, 1% penicillin/streptomycin).
5. Incubate the plates at 37 °C for 72–96 h until plaques become visible.
6. Fix the cell monolayers and stain with crystal violet (containing 3.7% methanol) for 2 h at room temperature.
7. Then, carefully examine each well under a microscope to count the visible plaques. Plaques should appear as clear zones against the stained cell monolayer. Record the number of plaques in each well.
8. Take the average plaque count from all wells. Multiply this average by the dilution used during viral culture supernatant preparation. Divide this result by the volume of virus applied to the cell monolayer (usually in mL). Example Calculation for PFU/mL: If the average plaque count is 50 plaques, and you used a 10-fold dilution of the viral culture supernatant with a volume of 0.1 mL applied to the cell monolayer: Average plaque count: 50 plaques Dilution factor: 10 Volume of virus applied: 0.1 mL
$$\text{PFU/mL} = (\text{Average plaque count}) \times (\text{Dilution factor}) / (\text{Volume of virus applied})$$
$$\text{PFU/mL} = (50 \text{ plaques}) \times (10) / (0.1 \text{ mL}) = 50,000 \text{ PFU/mL}$$
9. Calculate the MOI (Multiplicity of Infection) by dividing the PFU/mL by the number of cells infected. The number of cells infected can be determined by counting the total number of cells in a well before infection or by using a known cell density. Example MOI Calculation: If you have 1 million cells in a well and the PFU/mL is 50,000:
$$\text{MOI} = (\text{PFU/mL}) / (\text{Number of cells infected})$$
$$\text{MOI} = (50,000 \text{ PFU/mL}) / (1,000,000 \text{ cells}) = 0.05 \text{ MOI}$$

3.7 Virus infection on cells

3.7.1 Direct virus exposure on bEnd3 endothelial cells:

The objective of this experiment is to investigate the direct infection of blood-brain barrier (BBB) endothelial cells, specifically bEnd3 cells, with Japanese encephalitis virus (JEV), Dengue virus (DENV), and Rabies virus (CVS-11). This aims to simulate viral interactions with BBB endothelial cells, facilitating the study of potential neuroinvasive properties of these viruses. This experiment is significant as it allows researchers to assess the ability of JEV, DENV, and Rabies virus to infect BBB endothelial cells directly. Understanding how these viruses interact with the BBB is crucial for unraveling the mechanisms of viral neuroinvasion, which can lead to severe neurological diseases. By examining the impact of viral infections on BBB endothelial cells, researchers can gain insights into virus-host interactions and identify potential targets for therapeutic interventions aimed at preventing or mitigating neuroinvasive infections. This experiment involves three different viruses, enabling a comparative analysis of their infection mechanisms and effects on BBB cells. Such comparative studies can reveal unique characteristics of each virus and aid in the development of targeted interventions. Findings from this experiment may contribute to the development of strategies to prevent or treat neuroinvasive viral infections, ultimately benefiting public health and enhancing our ability to combat diseases caused by JEV, DENV, and Rabies virus.

1. Use bEnd3 cells as the substrate for the BBB endothelial cell model, procured from ATCC-USA.
2. Prepare the cell culture medium containing 7.5% Sodium bicarbonate and 10% Fetal Bovine Serum (FBS, Life Technologies, India).
3. Maintain the bEnd3 cells at 37°C under 5% CO₂ until confluence is reached.
4. Subculture the cells using mechanical disruption procedures/scraping (Corning, India).
5. Centrifuge the subcultured cells at 1500rpm for 10 minutes.
6. Split the cells in the ratio of 1:2 to 1:4 as per the experimental requirements.
7. Seed bEnd3 cells in a 25 cm² flask and incubate overnight in DMEM with 2% FBS.

8. Primary infect the cells with Japanese encephalitis virus, Dengue virus, and Rabies virus (MOI = 0.1).
9. Obtain the Dengue virus and Japanese encephalitis virus from National Institute of Virology, Pune, India (JEV GP-78, DENV2 #P23085).
10. Procure Rabies Virus Challenged Virus Strain (CVS-11) from CRI, Kausali.
11. Infect cells individually with JEV GP-78, DENV2, and CVS-11 in 5ml DMEM and 2% FBS.
12. Perform the virus infection for 2 hours in separate flasks and in triplicate.
13. Confirm the infection by qRT-PCR using SYBR-GREEN chemistry (Lanciotti et al., 1992).
14. Perform the qRT-PCR using the Applied Biosystems Inc. StepOne Plus Machine.
15. Collect the supernatant for protein analysis.
16. Process the cells for extraction of transmembrane protein.

3.7.2 Brain endothelial cells exposed to supernatants of virus exposed RAW cell supernatant:

The objective of this experiment is to investigate the immune response on brain endothelial cells and neuronal cells when exposed with supernatants of virus exposed murine monocyte (RAW) cell line. This study aims to understand how viruses, including JEV GP-78, DENV2, and CVS-11, can activate monocytes and subsequently affect the immune modalities and interaction dynamics with brain endothelial and neuronal cells. The significance of this experiment lies in its potential to elucidate the mechanisms of viral neuroinvasion and neuropathogenesis. By studying the virus-exposed monocytes effect on neuronal cells and brain endothelial cells, researchers can gain insights into the complex interplay between immune responses and viral infections in the central nervous system. This knowledge may contribute to

a better understanding of diseases like Japanese encephalitis, dengue, and rabies, potentially leading to the development of therapeutic strategies targeting neuroinvasive viruses.

1. Use the Murine monocyte (RAW) cell line as the model for the immune response stimulus.
2. Infect RAW cells with JEV GP-78, DENV2, and CVS-11 (MOI = 0.1).
3. Perform the virus infection for 2 hours individually with JEV GP-78, DENV2, and CVS-11 in separate flasks and in triplicate.
4. Collect the virus-activated RAW cell supernatant post-infection.
5. Overlay the virus-activated RAW cell supernatant on bEnd3 cells and neuronal-like SH-SY-5Y and N2a cell lines.
6. Expose the bEnd3 cells using the virus-activated monocyte supernatant after 2 hours of direct infection, incubating for a further 2 hours.
7. Propagate an identical set of control flasks.
8. Confirm the infection by RT-PCR using SYBR-GREEN chemistry (Lanciotti et al., 1992).
9. Perform qRT-PCR using the Applied Biosystems Inc. StepOne Plus Machine.
10. Collect the supernatant from bEnd3 and neuronal cells for protein analysis.
11. Process the cells for the extraction of transmembrane proteins.

3.8 MTT Cytotoxicity Assay

The objective of the MTT Cytotoxicity Assay is to assess the cytotoxic effects of JEV GP-78, DENV2, and CVS-11 viruses on various cell types, including RAW, bEnd3, SH-SY-5Y, and N2a cells, with the aim of understanding their potential role in disrupting blood-brain barrier tight junction proteins. By inoculating these viruses into different cell lines and measuring cytotoxicity, this experiment seeks to elucidate the impact of these viruses on cell viability, which may provide insights into their contribution to neuropathogenesis. The significance of this experiment lies in its ability to provide quantitative data on the cytotoxicity induced by

these neurotropic viruses in relevant cell models. Understanding how these viruses affect cell viability is essential for unraveling the mechanisms behind blood-brain barrier disruption and subsequent neuropathogenesis. This information may inform strategies for mitigating the neurological complications associated with viral infections, ultimately advancing our knowledge of virus-host interactions in the context of the blood-brain barrier.

1. Inoculate JEV GP-78, DENV2, and CVS-11 viruses at an MOI of 0.1 into RAW, bEnd3, SH-SY-5Y, and N2a cells in separate 96-well plates.
2. Maintain identical plates for JEV GP-78, DENV2, and CVS-11 virus-infected RAW cells for infection with supernatants of virus-exposed RAW cells. Use DMSO as a negative control.
3. Incubate all plates at 37°C with 5% CO₂ for 16-18 hours.
4. Add 100µl (10%) of 5mg/ml MTT solution to each well.
5. Incubate the plates for an additional 4 hours at 37°C.
6. Solubilize the formazan crystals formed by adding DMSO to each well.
7. Measure the absorbance at 550 nm using a Multimode ELISA reader.

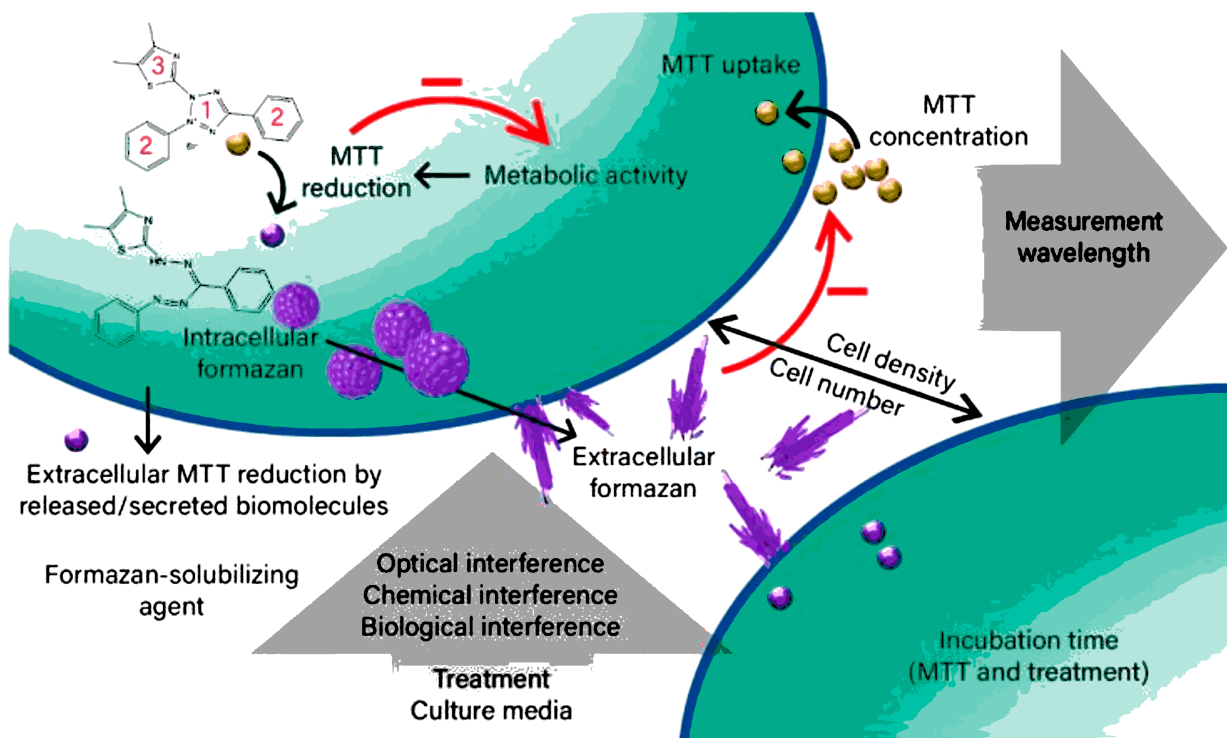


Fig 3 MTT Assay mechanism for determining cytotoxicity Source credit: Ghasemi, Mahshid et al. "The MTT Assay: Utility, Limitations, Pitfalls, and Interpretation in Bulk and Single-Cell Analysis." *International journal of molecular sciences* vol. 22,23 12827. 26 Nov. 2021, doi:10.3390/ijms222312827.

3.9 Cells RNA extraction

The respective viral RNA was extracted from cell culture supernatants harvested after successful virus propagation. Viral RNA Kit (Himedia, India) was used for the RNA extraction of respective viruses. Briefly, 10^6 - 10^8 cells were subjected to lysis and extraction as per manufacturer's protocol. This was reverse transcribed to cDNA (Thermo Scientific, India) and stored at -80°C until further analysis was carried out for studying gene expression.

3.10 Virus infected cells RNA isolation

The objective of this experiment is to isolate and purify viral RNA from infected cells, clinical specimens, and viral isolates using the QIAamp® Viral RNA Mini-isolation kit. This purified viral RNA will then be used for various molecular assays, including qRT-PCR and RT-PCR, to study the role of viruses in disrupting blood-brain barrier tight junction proteins, ultimately contributing to our understanding of neuropathogenesis. This RNA isolation process is a critical step in elucidating the molecular mechanisms underlying blood-brain barrier disruption by viruses. By isolating viral RNA, researchers can analyze the expression of cytokine genes and other factors involved in neuropathogenesis, providing valuable insights into the pathophysiology of neurological complications caused by viral infections. Additionally, this protocol ensures the availability of high-quality viral RNA for further studies, including the development of potential therapeutic interventions targeting virus-induced blood-brain barrier disruption.

1. Total RNA from all viruses infected cells was extracted using the QIAamp® Viral RNA mini isolation kit (Qiagen, India).
2. Briefly, 10^6 - 10^8 cells were subjected to lysis and extraction as per manufacturer's protocol.
3. The RNA was extracted manually using the QIAamp® Viral RNA mini kit (QIAGEN GmbH, Hilden, Germany) as per the manufacturer's instructions. QIAamp Viral RNA kit provides the fastest and easiest way to purify viral RNA for dedicated use in amplification technologies.
4. The kit uses the properties of a silica-gel-based membrane with centrifuge or vacuum technology.
5. The sample is first lysed under highly denaturing conditions to inactive RNases and also ensure for isolation of intact viral RNA.

6. Buffering conditions are then adjusted to provide optimum binding of the RNA to the silica gel-based membrane.
7. The RNA binds to the membrane and contaminants are washed with the two different wash buffers.
8. High quality purified RNA was eluted in a special RNase-free buffer; free of protein, nucleases, other contaminants and inhibitors.
9. The RNA was extracted from the clinical specimens and cell culture supernatant of virus isolates. The RNA extraction protocol as per the manufacturer's instruction was followed.
10. Preparations of reagents: Carrier RNA: 310 μL of buffer AVL was added to 310 μg lyophilized RNA to obtain a solution of 1 $\mu\text{g}/\mu\text{L}$. Ready to use AVL: 5.6 μL of carrier RNA was added to per 560 μL of AVL just before use. Buffer AW1: The supplied 95 mL concentrate was diluted with 125 mL ethanol (96-100%). Buffer AW2: The supplied 250 mL concentrate was diluted with 160 mL ethanol (96-100%).
11. Buffer AVL containing carrier RNA (1 $\mu\text{g}/\mu\text{L}$) was taken in 1.5 mL micro centrifuge tubes. Clinical specimens and viral isolates were thawed and 140 μL was added and incubated at room temperature.
12. A volume of 560 μL ethanol (96–100%) was added to the sample, mixed well by pulse vortexing for 15 seconds and centrifuged.
13. Next, 630 μL of the sample was loaded on to the membrane in the spin column, which was in turn placed in a 2 mL collection tube and was centrifuged at 8,000 rpm for 1 minute.
14. The tube containing the filtrate was then discarded and replaced with new tube and 500 μL of buffer AW1 was added onto the membrane and centrifuged at 8000 rpm for 1 minute.
15. The collection tube was discarded after every step. Further, 500 μL of Buffer AW2 was added to the above membrane and centrifuged at 14,000 rpm for 3 minutes.

16. The spin column was then placed in a clean micro-centrifuge tube and to the membrane 60 μ L of elution buffer AVE was added; incubated at RT for 1 minute and centrifuged at 8,000 rpm for 1 minute.
17. The eluted 60 μ L contained the viral RNA. The eluted RNA was used for qRT-PCR and RT-PCR while the remaining RNA was stored in a -80°C deep freezer. This was reverse transcribed to cDNA (Thermo Scientific, India) and stored at -80°C until further analysis was carried out for studying cytokine gene expression.

3.11 Gene Expression studies

The objective of the gene expression study is to investigate the impact of virus infection on various categories of genes, including inflammatory, regulatory, blood-brain barrier (BBB) breakdown, and reactive oxygen species (ROS) generation genes. This experiment aims to understand how viruses influence the expression of these genes, particularly those related to BBB integrity, and their potential role in disrupting tight junction proteins within the blood-brain barrier. By quantifying gene expression changes using Real-time RT-PCR and evaluating fold changes, this research seeks to elucidate the molecular mechanisms underlying virus-induced neuropathogenesis. The significance of this experiment lies in its potential to reveal the molecular pathways through which viruses disrupt the blood-brain barrier and compromise neurological function. Understanding the alterations in gene expression, especially in genes related to BBB breakdown, can provide valuable insights into the pathogenesis of neurological diseases caused by viral infections. This finding may contribute to the development of targeted interventions to protect and maintain the integrity of the blood-brain barrier, ultimately improving our ability to combat neuropathogenesis associated with viral infections. Three sets of gene expression were classified based on their functions: (i) Inflammatory genes, including interleukin-1 beta (IL-1 β), tumor necrosis factor alpha (TNF- α), interleukin-10 (IL-10),

interferon gamma (IFN- γ), and interleukin-18 (IL-18); (ii) Regulatory genes, such as monocyte chemoattractant protein-1 (MCP-1) and interleukin-6 (IL-6); (iii) BBB breakdown genes, including occludin, matrix metalloproteinases (MMP2, MMP9), and tissue inhibitor of metalloproteinase (TIMP1); and (iv) Reactive oxygen species (ROS) generation genes, such as inducible nitric oxide synthase (iNOS). A RealTime RT-PCR kit (Thermoscientific, India) was used to amplify the genes. The primer and cycling conditions were utilized as described elsewhere (Overbergh et al., 2003; Chen et al., 2011). The housekeeping gene GAPDH was employed for normalization. The Δ Ct value for each gene was calculated using three separate wells of all cell lines that were directly and indirectly infected with the respective viruses. To determine the fold change, the $\Delta\Delta$ Ct value was calculated using the mean Δ Ct value. A change was considered significant when the $\Delta\Delta$ Ct value was more than 2 (Schmittgen and Livak, 2008).

Table 1 cDNA preparation using RevertAid First strand cDNA synthesis kit:

Reaction Components	Reaction Volume
Template RNA	100 ng
Primer	1 μ L
NF water	Upto 11 μ L
5X reaction buffer	4 μ L
10mM dNTP Mix	2 μ L
Enzyme(200U/ μ L)	1 μ L
Total	20 μ L

Table 2 cDNA preparation temperature condition of thermal cycler:

Settings	Temperature	Time
Step 1	25°C	5 minutes
Step 2	42°C	60 minutes
Step 3	70°C	5 minutes
Step 4	4°C	∞

Table 3 Primers Sequence for *in-vitro* BBB disruption qPCR analysis:

Gene	Primer Sequence	Annealing Temperature	Amplicon Size
MMP-2- F	5'-TTCCCTAAGCTCATCGCAGACT-3'	55°C	196 bp
MMP-2-R	5'-CACGCTCTTGAGACTTTGGTTCT-3'		
MMP-9-F	5'-GCGTGTCTGGAGATTCGACTT-3'	55°C	229 bp
MMP-9-R	5'-TATCCACGCGAATGACGCT-3'		
iNOS-F	5'-GACATTACGACCCCTCCCAC-3'	55°C	254 bp
iNOS-R	5'-GCACATGCAAGGAAGGGAAC-3'		
TIMP-1-F	5'-GCAACTCGGACCTGGTCATAA-3'	55°C	226 bp
TIMP-1-R	5'-CGGCCCCGTGATGAGAAACT-3'		

IL-6-F	5'-GAGGATACCACTCCCAACAGACC-3'	56°C	221 bp
IL-6-R	5'-AAGTGCATCATCGTTGTTTCATACA3'		
MCP-1-F	5'-CTTCTGGGCCTGCTGTTCA-3'	55°C	285 bp
MCP-1-R	5'-CCAGCCTACTCATTGGGATCA-3'		
Occludin-F	5'-CACACTTGCTTGGGACAGAGG-3'	56°C	197 bp
Occludin-R	5'-TGAGCCGTACATAGATCCAGGAGC-3'		
IL-10-F	5'-GGTTGCCAAGCCTTATCGGA-3'	55°C	191 bp
IL-10-R	5'-ACCTGCTCCACTGCCTTGCT-3'		
IL-18-F	5'-ACTGTACAACCGCAGTAATACGG-3'	58°C	271 bp
IL-18-R	5'-AGTGAACATTACAGATTTATCCC-3'		
IL-1beta-F	5'-CAACCAACAAGTGATATTCTCCATG-3'	55°C	245 bp
IL-1beta-R	5'-GATCCACACTCTCCAGCTGCA-3'		
TNF- α -F	5'-CATCTTCTCAAAATTCGAGTGACAA-3'	55°C	242 bp
TNF- α -R	5'-TGGGAGTAGACAAGSTACAACCC-3'		
IFN- γ -F	5'-TCGCCGCGATCAAGGAGTTCTTCGGC-3'	58°C	201 bp
IFN- γ -R	5'-TGCACGTCGCGGACCTCCAGCGGCAC-3'		
GAPDH-F	5'-GCACAGTCAAGGCCGAGAAT-3'	55°C	211 bp
GAPDH-R	5'-GCCTTCTCCATGGTGGTGAA-3'		

Table 4 Temperature conditions for *in-vitro* BBB disruption qPCR analysis:

CYCLE STEP	TEMPERATURE	TIME	CYCLES
Initial Denaturation	95°C	30 secs	1
Denaturation	95°C	5 secs	40
Annealing	#55°C	30 secs	
Extension	72°C	30 secs (*🔥 plate read)	
Melt Curve	60-95°C	various	1

*Optics were kept open.

#Annealing temperature as mentioned in above Primer sequence Table No

(B) *In-vivo* studies in *Drosophila melanogaster* glial-brain barrier

3.12 *Drosophila* breeding, maintenance

1. Fly Storage: All fly handling will take place in the invertebrate lab or the invertebrate prep room. Keypad locks control entry to these rooms. The doors to both rooms must be kept always closed.
2. Fly vials containing live genetically modified flies will never be opened outside of these rooms, so that the potential for escapes is minimized.
3. Flies are kept in stoppered vials placed in racks, or stoppered bottles placed in trays, in incubators ranging from 18° to 29°C. Some fly stocks may also be kept at room temperature on shelving or benches. It may be necessary to transfer fly vials between these rooms. When doing so careful attention must be paid to ensure that stoppers are secure, and that bottles and vials are secured in appropriate racks.

4. Secondary containment should also be used, e.g., placing racks inside a plastic bag or box.
Do not try to carry too much; this is when accidents happen, use of a trolley is recommended. If two trips are necessary, make two trips.
5. Flies will be removed to other locations only in proper double containment (either: 1, capped vials or bottles enclosed in a plastic bag to be taken directly to the autoclave or the freezer; or 2. capped vials enclosed in a box for shipping).
6. CO₂ anesthesia: For many procedures flies must first be anaesthetise by flooding the vial with CO₂. Anesthetize flies are then placed onto a pad where CO₂ is delivered at a slow flow rate sufficient to keep flies anaesthetised for as long as necessary.
7. Anaesthetised flies are manipulated using soft brushes and the required flies are selected for transfer to a fresh vial or for sample collection.
8. Un-needed anaesthetised flies are immediately placed into bottles containing water with a few drops of washing up liquid added (fly morgues) to kill them.
9. Fly morgues must be changed on a weekly basis. To do so the water is strained through paper towels and dead flies removed to an autoclave bag.

3.13 *Drosophila* cross setup

Before starting with crossing genetic fly lines with reporter lines. We crossed the unbalanced fly lines with GFP, Pr, Tm6B, and Sb markers. The ideal breeding temperature for flies is at 25°C but UAS-mac-NOS lines weren't producing any correct marked progenies. Therefore, we tried breeding them at 18°C, through which we could obtain correct transgenic progenies from some of the crosses.

1. Crosses for up-regulation of NOS:

Table 5 Crosses between genetic and reporter lines of *Drosophila* for up-regulation of NOS

Type of Glia	Genetic lines	Reporter line
All glia	repo-G4 (precursor process need balancer)	w-; S/CyO;UAS-mac-NOS/Tm6b
Perineurial glia	w-; NP6293-Gal4; UAS-GFP/CyO	
Sub-perineurial glial	Moody-G4 (precursor process need balancer)	

2. Crosses for down-regulation of NOS:

Table 6 Crosses between genetic and reporter lines of *Drosophila* for down-regulation of NOS

Type of Glia	Genetic lines	Reporter line
All glia	repo-G4 (precursor process need balancer)	UAS-NOS-RNAi #50675
Perineurial glia	w-;NP6293-Gal4;UAS-GFP/CyO	
Sub-perineurial glial	Moody-G4 (precursor process need balancer)	

3.14 *Drosophila* fly brain RNA extraction

1. In this experiment, gene expression studies were conducted using fruit fly heads. A total of 50-55 heads were dissected for each transgenic correct progeny.
2. The heads were immediately processed for RNA extraction using the NEB Monarch RNA isolation kit.
3. The RNA extraction process was carried out according to the protocol provided by the kit manufacturer.
4. The NEB Monarch RNA isolation kit was used to extract RNA from the dissected fly heads. The kit protocol involved lysis of the head tissue using a lysis buffer, followed by RNA binding to a silica membrane in the presence of high concentration of chaotropic salt.
5. After washing the membrane to remove contaminants, RNA was eluted from the membrane with a low salt elution buffer.
6. The RNA was then subjected to quality control and quantification using a spectrophotometer. The resulting RNA samples were used for downstream gene expression studies.

3.15 *Drosophila* fly brain gene expression

Table 7 cDNA preparation using First strand cDNA synthesis:

Reaction Components	Reaction Volume
10X reaction buffer	2 μ L
25X dNTP Mix (100mM)	0.8 μ L
10X RT Random Primer	2uL
NF water	Upto 4.2 μ L
Enzyme(20U/ μ L)	1 μ L
	10 μ L

Add the above component on an ice-chiller in the given order to make 10uL of 2X master mix. To this add 10uL of extracted RNA samples in respective tubes. Mix gently and centrifuge the reaction tubes before putting in thermal cycler. For random hexamer primers 5 mins at 25°C followed by 37°C for 60 mins. Stop the reaction at 70°C for 5 mins. Use it immediately for qPCR step or store at -20°C for 1 week.

Table 8 Primer Sequence for gene expression studies in *Drosophila*

Gene	Primer Sequence	Annealing Temperature	Amplicon Size
iNOS-F	5'-ACCAGACATCCCATGTGAAGC-3'	56°C	218 bp
iNOS-R	5'-GCCTGTGTAATGGAACCATACCA-3'		
dmMMP1- F	5'-TCAGTGTTTCATAGTCGTAGGCA-3'	56°C	159 bp
dmMMP1-R	5'-GGTAGATAGCCGAACCTGGGAC-3'		
dmMMP2- F	5'-AAAAACCGCTATCTCCGAGGA-3'	56°C	148 bp
dmMMP2-R	5'-TGTAATGTTGCCAAAAGACTGGA-3'		
Moody-F	5'-TATCATCAGCCTCTGTATTGCCG-3'	56°C	150 bp
Moody-R	5'-AGGGATACCCCTATGTTTCCG-3'		
NrxIV-F	5'-CAACTCCGATGGCAACTCTATC-3'	56°C	124 bp
NrxIV-R	5'-CCACACGCATGGAAATACGAT-3'		

Table 9 cDNA preparation temperature condition for *Drosophila* model:

Settings	Temperature	Time
Step 1	25°C	5 minutes
Step 2	37°C	2 x 60 minutes
Step 3	85°C	5 minutes
Step 4	4°C	∞

3.16 Gene expression studies on *Drosophila* fly brain extracted RNA

1. Prepare cDNA of interest using desired DNA extraction and purification method. Run each cDNA standard and sample in triplicate.
2. Thaw Luna Universal qPCR Master Mix and other reaction components at room temperature, then place on ice.
3. After thawing completely, briefly mix each component by inversion, pipetting or gentle vortexing.
4. Determine the total volume for the appropriate number of reactions, plus 10% overage and prepare an assay mix of all components except DNA template accordingly.
5. Mix thoroughly but gently by pipetting or vortexing. Collect liquid to the bottom of the tube by brief centrifugation.
6. Aliquot assay mixed into qPCR tubes or plates. For best results, ensure accurate and consistent pipetting volumes and minimise bubbles.
7. Add DNA templates to qPCR tubes or plates. Seal tubes with flat, optically transparent caps; seal plates with optically transparent film.

8. Care should be taken to properly seal plate edges and corners to prevent artefacts caused by evaporation. Spin tubes or plates briefly to remove bubbles and collect liquid (1 minute at 2,500–3,000 rpm).
9. Program the BIO-RAD CFX96 real-time instrument with below indicated thermocycling protocol. Ensure a plate read is included at the end of the extension step. Use the SYBR[®] or SYBR/FAM scan mode setting on the real-time instrument.

Table 10 Luna[®] Universal qPCR Master Mix Protocol for *in-vivo* BBB disruption analysis:

COMPONENT	20 µl REACTION	FINAL CONCENTRATION
Luna Universal qPCR Master Mix	10 µl	1X
Forward primer (10 µM)	0.5 µl	0.25 µM
Reverse primer (10 µM)	0.5 µl	0.25 µM
Template cDNA	variable	< 100 ng
Nuclease-free Water	to 20 µl	

Table 11 Thermal cycling conditions for gene expression studies in *Drosophila*:

CYCLE STEP	TEMPERATURE	TIME	CYCLES
Initial Denaturation	95°C	30 secs	1
Denaturation	95°C	5 secs	40
Annealing	56°C	30 secs	
Extension	72°C	1 min (*🔥 plate read)	
Melt Curve	60-95°C	various	1

*Optics were kept open

3.17 Immunological studies nrx4, moody-GFP protein studies

The procedure for *Drosophila* brain dissection and immunostaining is described in details in the following papers:

- 1) A protocol for dissecting *Drosophila melanogaster* brains for live imaging or immunostaining (2006) Nat. Protoc. 1, 2110–2115.
- 2) Improved and expanded Q-system reagents for genetic manipulations (2015) Nature Methods 12, 219–222.

Brains of adult flies are dissected in PBS in a watch glass, fixed in 4% PFA for 20 min at room temperature on a nutator, washed at room temperature in PBT (PBS+0.3% Triton-X100) for 5–6 h, blocked in 5% NGS in PBT and placed in primary antibody mixes for three nights at 4 °C

(cold room) on a nutator. Next, the brains are washed for several hours in PBT at room temperature and placed in secondary antibodies mix for two nights at 4 °C. The following day the brains are washed in PBT and placed in mounting solution (Slow Fade Gold or Vectashield) overnight at 4 °C without agitation, and mounted on a microscope slide the next day, sealed with nail varnish.

Confocal Microscopy Protocol for GFP-Fly Brain:

- A) Processing of sample (Fly Brain)
- B) Imaging of fixed mounted sample

(A) Fly Brain sample processing:

DAY 1

Dissect the brain in chilled 1X PBS (For each brain it should take less than 6-10 mins). Remove trachea, optical parts (orange eye) make the brain as clean as possible. Put the brain directly in (approximately 200-500 µL) 4%PFA in PBT freshly prepared at room temperature or freshly thawed a frozen vial of PFA. Do not try to freeze the vial again for further use Once the vial is thawed it should be used directly. Keep this (Brain+PFA) vial on a shaker for sharp 20 mins only for fixation at room temperature, dark (Cover with a black opaque tip box or something). After 20 mins, wash the brains with PBT for 5-6 hours. Remove the 4%PFA and immediately add PBT (approximately 200-500 µL) and keep it on the shaker for 30 mins, 35 rpm at room temperature, dark. After 30 mins remove PBT and add fresh PBT and keep the vials on the shaker. Repeat this procedure 3-4 times. Block fly brain in 5%NGS in PBT for at least 30 mins

at room temperature. Remove the final PBT and add approx. 200ul of Blocking solution in each vial and let it incubate at room temperature for 30 mins, 35 rpm, dark.

Table 12 Preparation of Blocking solution for confocal microscopy of *Drosophila*

BLOCKING SOLUTION			
NGS	5%	5 μ L	30 μ L
PBT	Up to	95 μ L	570 μ L
		100 μ L	600 μ L

After blocking for 30 mins, place the brain in Primary Antibody mix for three nights at 4°C (in cold room). The primary antibody Mouse nc82 stains the Brp protein (Bruchpilot – German for Crash Pilot) in red. The Brp protein is ubiquitously expressed in *Drosophila* brain and more localised to the neuropile region (Knappek, Stephan et al., 2009). While the Rabbit anti-GFP primary antibody stains the moody protein in the sub-perineural glia barrier of the *Drosophila* brain in fluorescence green.

Table 13 Protocol for the preparation of Primary Antibody

PRIMARY ANTIBODY MIX			
Mouse nc82 (DSHB, 1:25)	4%	4 μ L	24 μ L
Rabbit anti-GFP (Life Technologies # A11122, 1:100)	1%	1 μ L	6 μ L
NGS	5%	5 μ L	30 μ L
PBT	Up to	90 μ L	540 μ L
		100 μ L	600 μ L

DAY 4

Wash brains for several hours in PBT at room temperature. Remove the Primary antibody mix and add PBT (approximately 200-500 μ L) and keep it on the shaker for 30 mins, 35 rpm at room temperature, dark. After 30 mins remove PBT and add fresh PBT and keep the vials on the shaker. Repeat this procedure 3-4 times. Remove the PBT in the last wash and add (approximately 200-500 μ L) of Secondary Antibody Mix for two nights (Day 4 - Day 5) at 4°C (in a cold room).

Table 14 Protocol for the preparation of Secondary Antibody

SECONDARY ANTIBODY MIX			
Alexa 488 anti-rabbit (Invitrogen #A11034)	0.5%	0.5 μ L	3 μ L
Cy3 anti-mouse (Jackson ImmunoResearch #115-165-062)	0.5%	0.5 μ L	3 μ L
NGS	5%	5 μ L	30 μ L
PBT	upto	94 μ L	564 μ L
		100 μ L	600 μ L

DAY 6

Wash brains for several hours in PBT at room temperature. Remove the Secondary antibody mix and add PBT (approximately 200-500 μ L) and keep it on the shaker for 30 mins, 35 rpm at room temperature, dark. After 30 mins remove PBT and add fresh PBT and keep the vials on the shaker. Repeat this procedure 3-4 times. Place the brain in a mounting solution (Slow Fade Gold) overnight at 4°C (in a cold room).

DAY 7

Mount brains on a microscopic slide. For imaging these slides follow the below mentioned protocol.

(B) Imaging of fixed mounted sample:

The SMC 2009 and Power Supply should be turned on, and the System Switch should be turned on. The CPU should be turned on, and the user should log on as LSM user. The Components Switch should be turned on, and the Laser Key should be turned on. The Zen Blue 2.6 System Software should be started, and +Zen System should be selected. For sample imaging, the Magnification Lens (Airy Green 20X) should be selected, and the Top button should be pressed for Stage Up, and the Bottom Button should be pressed for Stage Down. The slide should be placed, and the rod should be pushed in to see with the eye lens or pulled out to see the LSM confocal image. Locate should be clicked, and Transmitted Light/GFP/RFP should be selected. Then, the brain sample should be focused on, and the GFP/DAPI filter should be clicked. The sample (brain) should be checked again, and it should be focused. Acquisition should be clicked, and Smart Setup should be clicked. The experiment should be conFigd, and smartest Line should be selected, and OK should be clicked. Channels should be clicked, Track 1 on Imaging Setup should be clicked, and EGF [1AU] should be clicked. Live should be clicked, and Acquisition Mode should be set to Frame. The area should be cropped or scanned, and the image should be realigned on the screen. Pixels (Pre-set) such as 128x128, 256x256, or Optimal (e.g., 5172x5172) should be selected. The scan speed should be set to LOWEST or HIGHEST, and the direction should be set to Uni or Bi. Averaging should be set to 0|2x|4x|, and bits per pixel should be set to 8 or 16. For display, [Min/Max] should be clicked, and the Range Indicator should be clicked to decrease the master gain (v) and decrease the red dots. The Laser

Power should be kept constant at 0.2%, and the gain should be increased to 530-750V. For Z stack, First should be set by pressing Ctrl and keeping scrolling up & down with the mouse until the beginning of the top edge of the brain is reached. Last should be set by pressing Ctrl and keeping scrolling up & down with the mouse until the beginning of the bottom end of the brain is reached. Optimal Slicing or Manual Setup can be chosen, and Start Experiment should be clicked. After completing the Z-Experiment, Gallery should be clicked, and 3D should be clicked for viewing the 3D images. Save Image should be clicked. For Tile, X-1 and Y-3 should be selected, and X1 (only one x-axis plane) should be clicked to start the experiment. For processing, Stitching should be clicked, Fuse should be clicked, and ALWAYS Apply in [NEW OUTPUT] file should be clicked. The NewOutput stitched file should be saved in .tiff format.

3.18 Transmission Electron Microscopy of *Drosophila* fly brain

The protocol for the Transmission electron microscopy experiment on *Drosophila* fly brains is as follows dissect at least 6-10 brains from each of the following groups: Moody-NOS-RNAi, Moody-NOS-NOS, Moody-UAS-mac-NOS, and Moody-Parent the BBB.

1. Rapidly dissect brains into cold fly saline, remove any air sacs to prevent the brains from floating, and place the brains in 1X chilled PBS on a chilled watch glass.
2. Remove air sacs, trachea, and possibly the orange optical portion of the fly eye to prevent the brains from floating and entangling with trachea.
3. Fix the brains by immersing them in 2% paraformaldehyde + 2.5% glutaraldehyde in 0.1M sodium cacodylate for 1 hour at 4°C, followed by 1 hour at room temperature.
4. After dissection, immediately transfer each brain to a 10ml glass vial containing fixative solution.
5. Do not keep the glass vials on ice, let them remain at room temperature until all the fly brains are dissected.

6. Place the glass vials on a 360° rotator and let them incubate at room temperature for 1 hour, keeping the rotation speed at 4rpm.
7. Rinse the brains in 0.1M sodium cacodylate 3 times for 5 minutes each time, using glass pipettes/droppers to give any subsequent washes.
8. Remove the fixative solution without drying up the brain and add 0.1M sodium cacodylate solution, then place it back on the rotator for washing at 4rpm.
9. Post-fix the brains with 1% OsO₄ in 0.1M sodium cacodylate containing 0.1M immediately after removing the last wash 0.1M sodium cacodylate. Ensure that the volume is sufficient to immerse all the brains well.
10. Incubate the fly brains in an Osmium fixative solution for 1 hour on a rotator machine rotating at 4rpm.
11. After fixation, wash the specimens in 0.1M sodium cacodylate for 3 times for 5 minutes each time, using glass pipettes/droppers.
12. Dehydrate the specimens through an ethanol series (30%, 50%, 70%, 90%, and 100%) for 5 minutes each time.
13. Then, infiltrate the specimens with a 1:1 mixture of propylene oxide and Epon resin for 1 hour. Infiltrate the specimens with a 2:1 mixture of propylene oxide and Epon resin for 1 hour. Infiltrate the specimens with pure Epon resin overnight.
14. Embed the specimens in Epon resin in flat embedding molds and polymerize the resin in an oven at 60°C for 48 hours. Cut 70-90nm sections using a diamond knife and place them on copper grids.
15. Stain the sections with 2% uranyl acetate for 15 minutes, followed by lead citrate for 5 minutes. Examine the sections using a transmission electron microscope, and capture images of the Moody-NOS-RNAi, Moody-NOS-NOS, Moody-UAS-mac-NOS, and Moody-Parent the BBB groups of *Drosophila* fly brains.

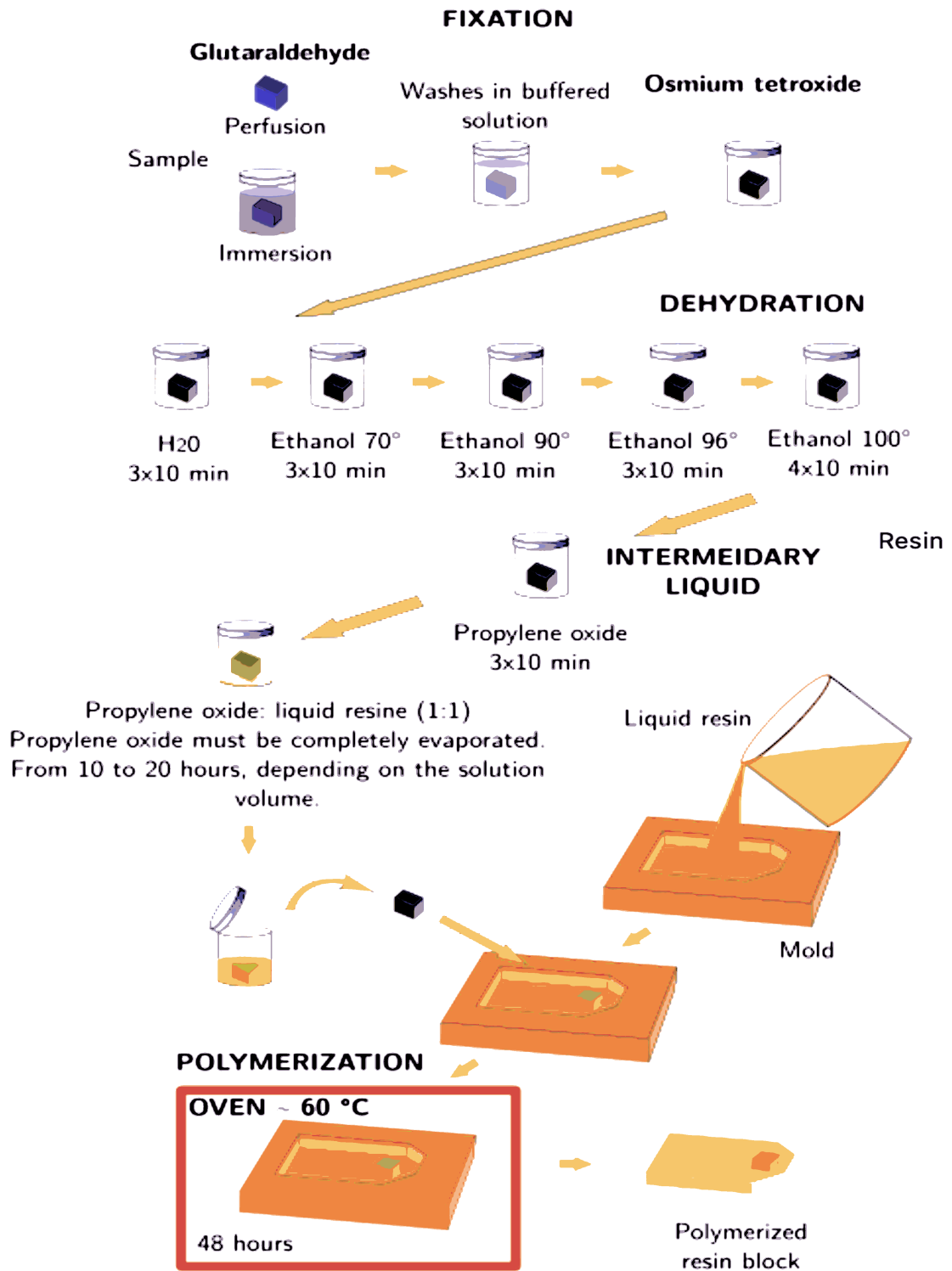


Fig 4 Flow method for fixation and embedding of *Drosophila* fly brain for TEM imaging

Statistical Analysis

Data are expressed as means $\pm$ SEM, and the significance of differences between groups was evaluated by using a two-tailed Student *t* test or one-way analysis of variance, followed by Dunnett multiple comparison test. Graphs were plotted and analyzed using GraphPad Prism10 (GraphPad, La Jolla, CA). Asterisks represent significant differences, compared to control.; ns, not significant * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$.

Chapter 4

Results

4.1 In silico studies of dengue envelope protein

Dengue Envelope Protein (DEP) structure construction (SWISSMODEL)

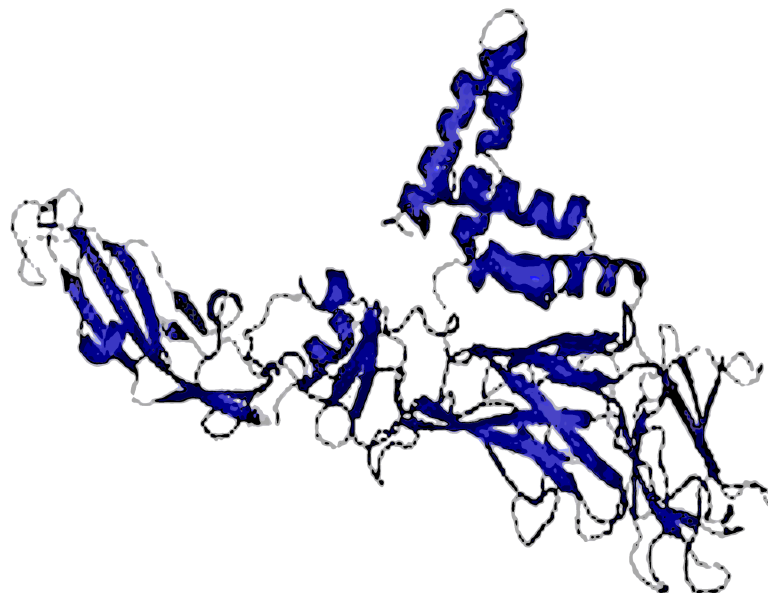


Fig 5 Dengue Envelope Protein (DEP, 3j27.1.C) obtained from SWISS-MODEL

After submitting the Dengue envelope protein sequence in SWISS-MODEL software, we obtain the above energy minimized stable structure. This structure was further subjected to protein-protein interaction with brain membrane proteins using PATCHDOCK and FIREDOCK soft wares. The binding energy obtained after PATCHDOCK and FIREDOCK are mentioned in below table.

Table 15 The comparison of lowest energy pose of Dengue envelope protein with other viruses mentioned in literature.

Receptor	Binding Energy for DEP (kJ/mol)	Reported Binding Energy (kJ/mol)
Voltage dependent P-Q type calcium channel subunit alpha 1A	-17.26	-53.12
Vacuolar protein sorting associated protein-13C	-49.60	-45.13
Cytoplasmic dynein 1 heavy chain 1	-45.12	-50.67
Plasma lemma vesicle associated protein	-38.72	-29.27
Nesprin1	-36.31	-44.73
Brefeldin-A inhibited guanine nucleotide exchange protein-2	-36.15	-136.18
Annexin A6	-24.81	-19.56
Transcription factor SOX-5	-23.95	-50.81
Rabenosyn-5	-21.00	-84.19
Voltage dependent P-Q type calcium channel subunit alpha 1A	-17.26	-53.12

**The lowest binding free energy found in literatures of different viruses such as WNV, JEV and other Flaviviruses*

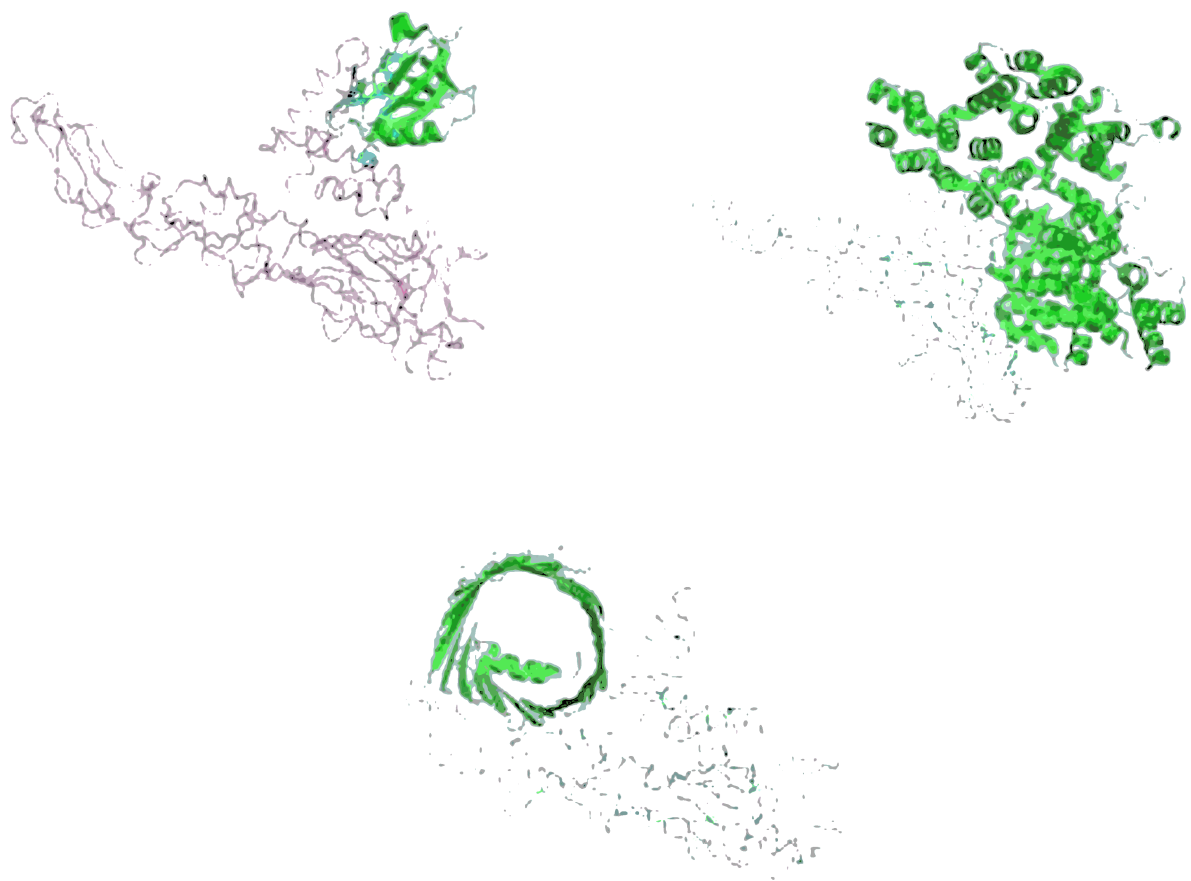


Fig 6 In-silico virus envelope protein- brain endothelial transmembrane receptor interaction analysis (a) DEP-ANXA6 Annexin A6 brain membrane receptor interactome (b)DEP-VP13Cvacuolar protein sorting associated protein-13Cbrain membrane receptor interactome (c) PLVAP Plasma lemma vesicle associated protein brain membrane receptor obtained from FireDock server.

4.2 Virus Propagation

4.2.1 Dengue virus

The CPE was observed at around day 6 post infection and onward, which consisted of enlarged infected cells fused with the neighbouring infected cells and appearance of vacuoles within cell cytoplasm. Following Fig shows control (uninfected) C6/36 cells and DENV infected C6/36 cells.

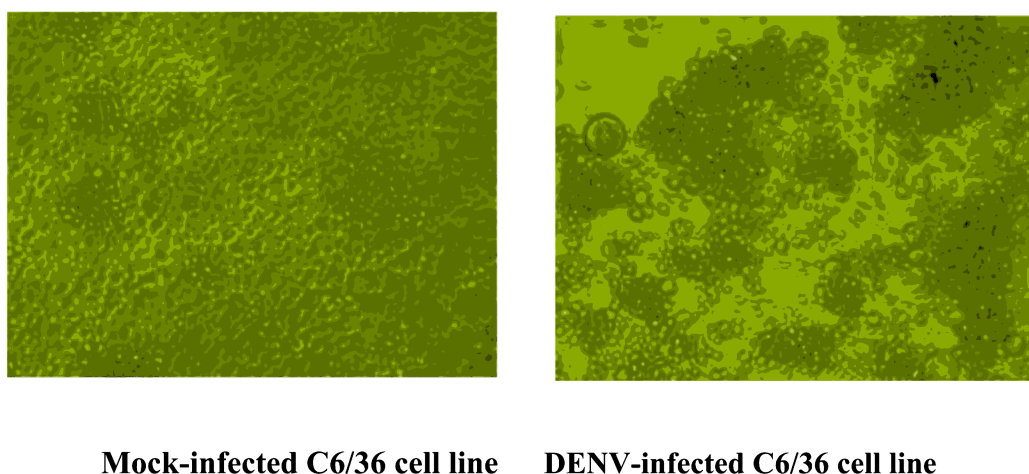


Fig 7 C6/36 cell lines uninfected and infected with Dengue virus type 2

Tissue culture infectivity dose 50-CPE (TCID₅₀)-CPE titre was determined by Reed and Muench method using C6/36 cell line and serial dilutions of DENV-2. The TCID₅₀-CPE titre for DENV-2 serotype was found to be 10⁵/ 100μl.

4.2.2 Japanese Encephalitis Virus

Porcine stable cell lines were overlaid with JEV GP-78 virus with 2% Fetal Bovine Serum with control mock infected flask. After completion of incubation period, the cell clusters were observed indicating the alleviation in virus replication in cells (can be observed in the below image).

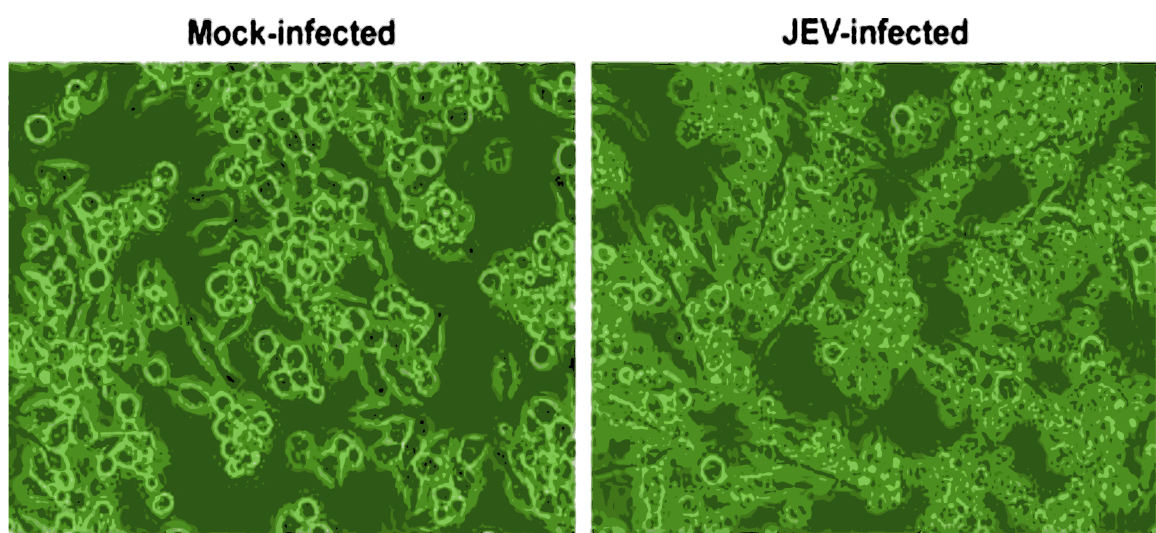
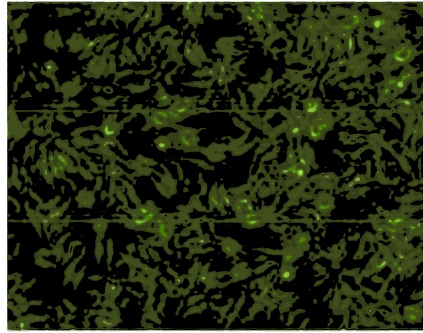


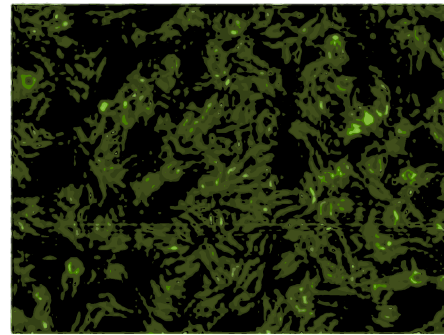
Fig 8 Porcine Stable cell lines uninfected and infected with JEV-GP78 virus

4.2.4 Rabies Virus

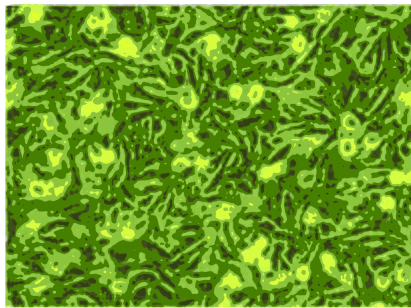
Post 24-96 hours after incubation of Rabies Virus (CVS-11) propagation in BHK-21 cells was observed.



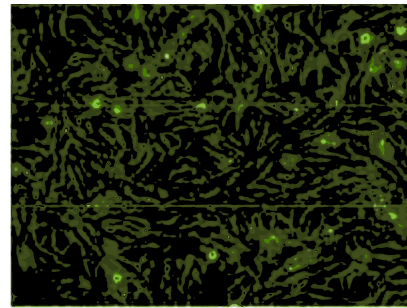
BHK-21 infected with CVS after 24 hrs



BHK-21 infected with CVS after 48 hrs



BHK-21 infected with CVS after 72 hrs

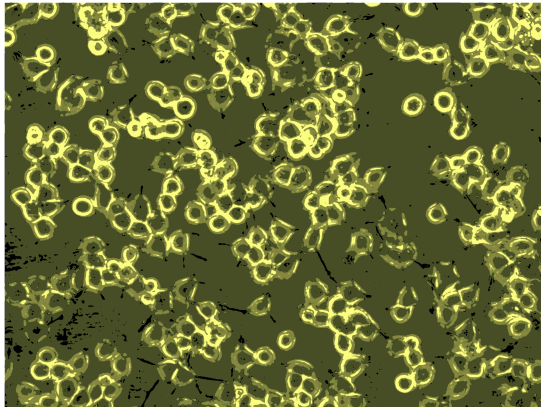


BHK-21 infected with CVS after 96 hrs

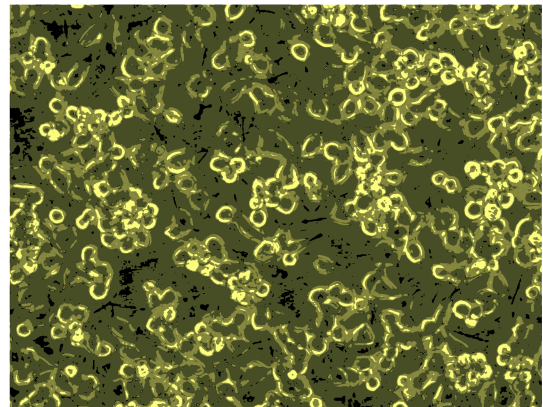
Fig 9 BHK21 cell lines uninfected and infected with CVS-11 rabies virus

And when we look at the morphological changes in BV2 cells following lipopolysaccharide stimulation, the non-stimulated or unstimulated BV2 cells, a commonly used microglial cell line, appeared diverse in terms of shape and structure. Without any external stimulus, the cells displayed a range of morphologies, from spherical to ameboid, and some cells even had protrusions. However, upon exposure to LPS (100ng/mL), a well-established pro-inflammatory stimulus, the cells underwent a significant change in their appearance. The protrusions on the cells were greatly reduced and the overall shape of the cells became more spherical or flattened.

Additionally, if the cells had "spikes" present, they were observed to be thickened and shortened as shown in the figure below. This change in cell morphology after LPS exposure is likely a result of the inflammatory response induced in the cells.



Mock-Infected



Infected

BV2 (Murine microglial) cells grown in DMEM with FBS (Mock-Infected), cells show heterogeneous morphology with presence of prominent protrusions and BV2 cells 4 hours after exposure to LPS cells show distinct flattening and increased appearance of spherical cells, with reduction in the number of spikes.

4.3 Cytotoxicity Analysis on virus on cells:

The viral apoptosis upon direct infection with JEV GP-78, DENV2 and CVS-11 and with supernatants of virus-exposed murine monocytic cells on brain endothelial cell lines (bEnd3) and neuronal cell lines (SH-SY-5Y and N2a). The cytotoxicity assay was analysed for bEnd3 exposed to supernatants from individual virus activated murine monocyte cells (RAW) post 2

hours of infection. The dimethyl sulfoxide (50 μ M) was kept as an control in all the group of experiments.

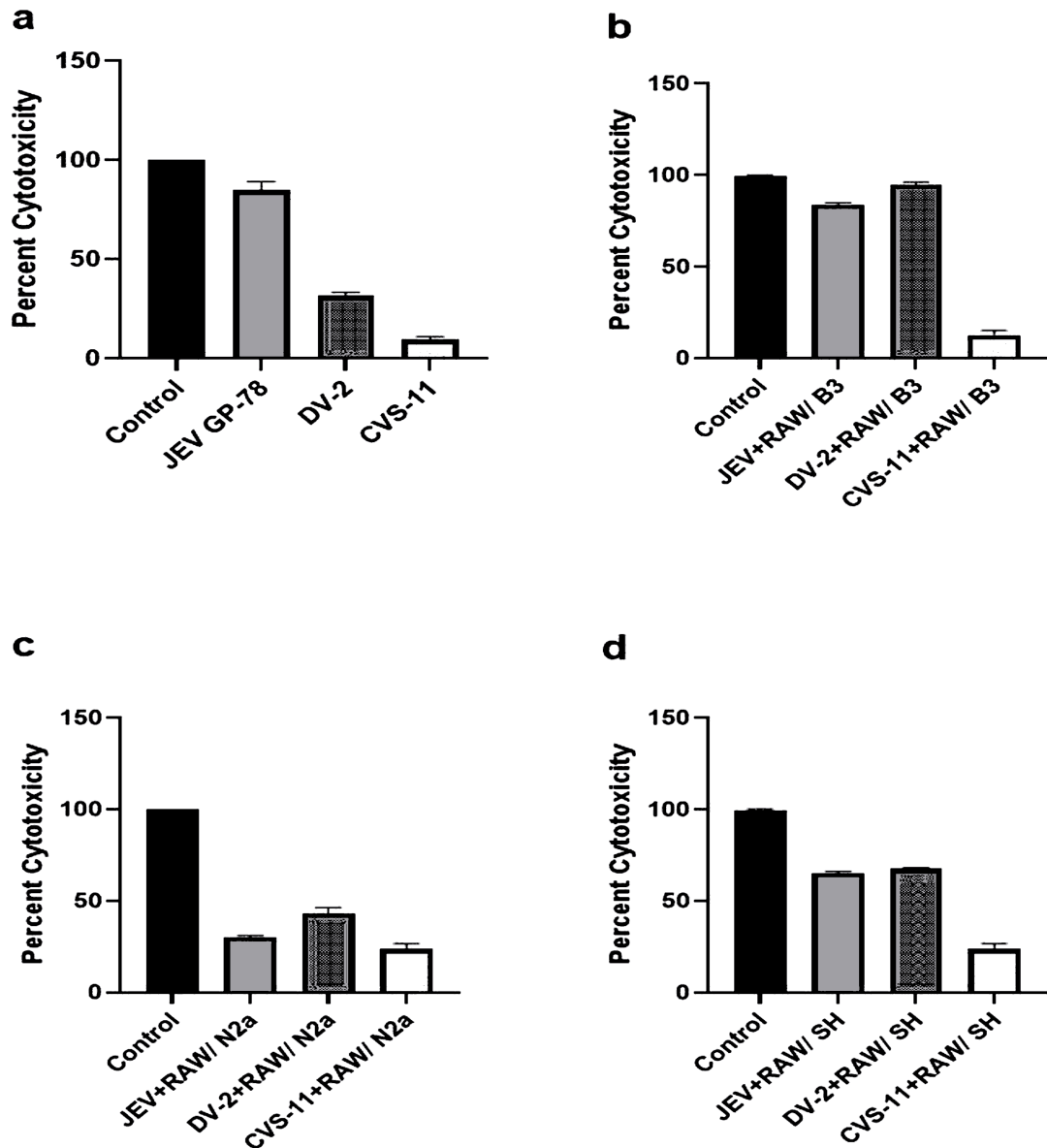


Fig 10 Percentage cytotoxicity observed on bEnd3, N2a and SH-SY-5Y cells when infected with JEV GP-78, DENV2 and CVS-11. a) when viruses directly exposed to bEnd3 cells b) bEnd3 cells exposed to supernatant from virus exposed to RAW cells c) N2a cells exposed to supernatant from virus exposed to RAW cells d) SHSY5Y cells exposed to supernatant from virus exposed to RAW cells. DMSO was used as a positive control for toxicity.

4.4 Gene expression analysis of *In-vitro* studies on bEnd3 cells

The bEnd3 cells were harvested post exposure with JEV GP-78, DENV2, CVS-11 and the supernatant from these viruses exposed to murine monocyte. Then, total RNA was extracted to confirm the extent of disruption of BBB tight junction proteins might cause due to the alleviation of inflammatory cytokines. The gene expression analysis was carried out using Applied Biosystems Inc. GAPDH was kept as endogenous reference control. The mean fold change and the standard deviation is mentioned below. In this study, we investigated the gene expression levels of various key genes IFN-gamma, IL-6, IL-10, IL-1beta, IL-18, iNOS, MMP-2, MMP-9, Occludin, TIMP-1, and TNF-alpha in bEnd3 cells following exposure to different viruses. The gene expression data were analysed statistically using a One-way ANOVA followed by Dunnett multiple comparison test. The One-way ANOVA test was chosen to determine if there were any significant differences in gene expression levels among the groups exposed to different viruses. This test is appropriate for comparing means across multiple groups (in this case, different viruses) simultaneously. It helps identify whether the means of gene expression levels are statistically different and if the variations observed are due to the exposure to the respective viruses. The results of the statistical analysis revealed significant variations in the expression levels of each gene when compared to the control group. This suggests that exposure to different viruses indeed influences the gene expression profiles in bEnd3 cells. The means $\pm$ SEMs from three independent replicates were calculated for each gene, providing a comprehensive understanding of the effects of viral exposure on the expression of these crucial genes.

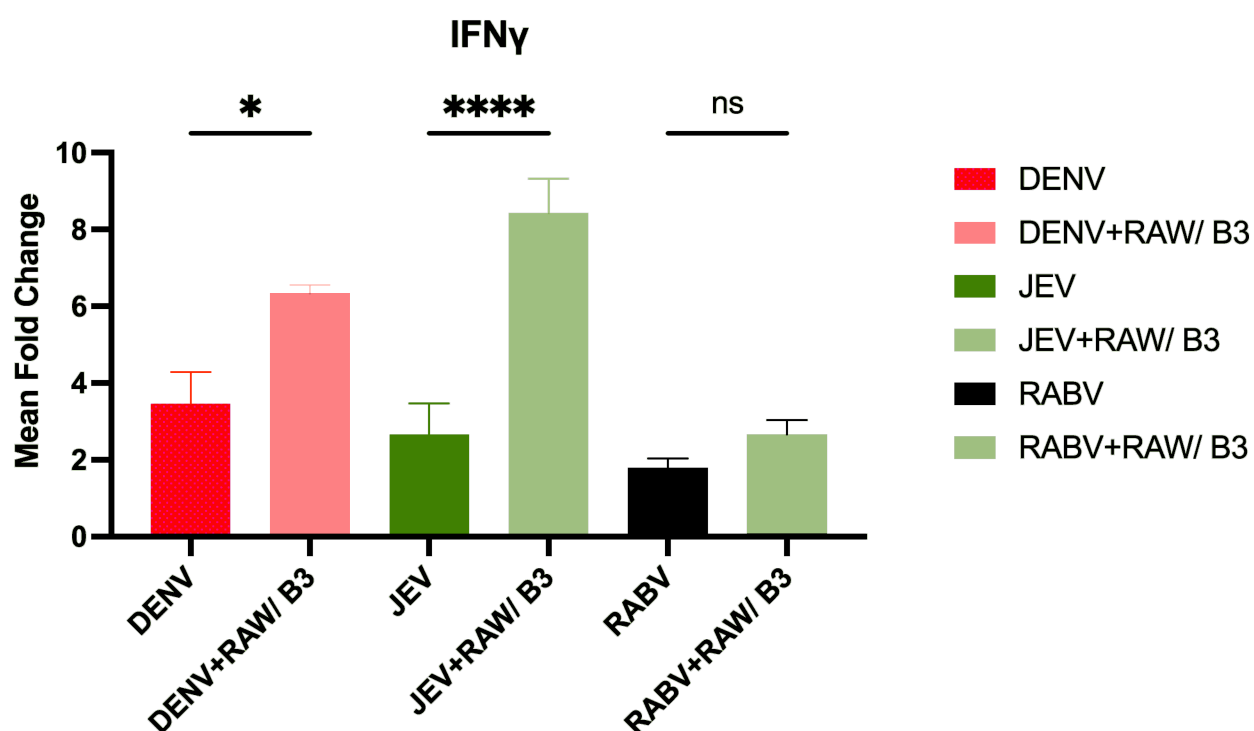


Fig 11 Expression of IFN-gamma in the bEnd3 cells post-exposure to different viruses.

The results reflect the means $\pm$ SEMs from three different replicates. (Asterisks represent significant differences, compared to control.; ns, not significant * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$.)

Table 16 Gene expression of IFN-gamma after virus exposure to bEnd3 cells

Group	Mean Fold Change
DENV	3.46
DENV+RAW/B3	6.34
JEV	2.66
JEV+RAW/B3	8.43
RABV	1.79
RABV+RAW/B3	2.66

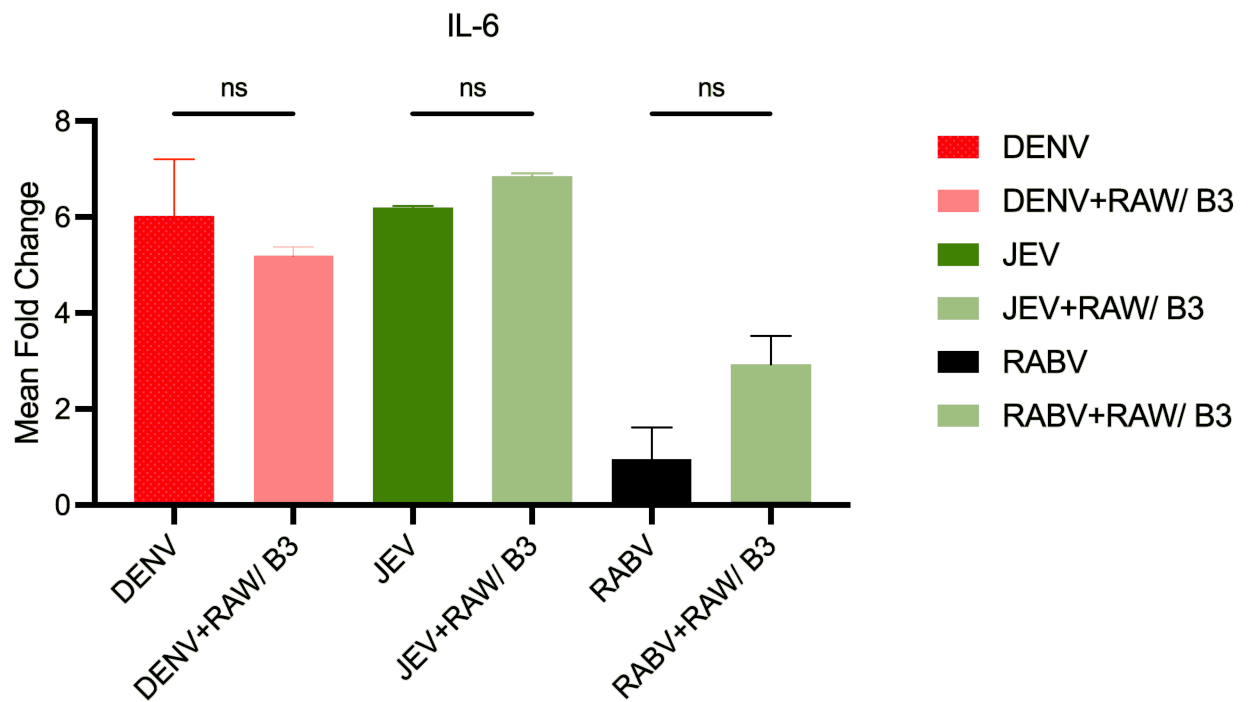


Fig 12 Expression of IL-6 in the bEnd3 cells post-exposure to different viruses. The results reflect the means $\pm$ SEMs from three different replicates. (Asterisks represent significant differences, compared to control.; ns, not significant * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$.)

Table 17 Gene expression of IL-6 after virus exposure to bEnd3 cells

Group	Mean Fold Change
DENV	6.01
DENV+RAW/B3	5.19
JEV	6.19
JEV+RAW/B3	6.84
RABV	0.95
RABV+RAW/B3	2.93

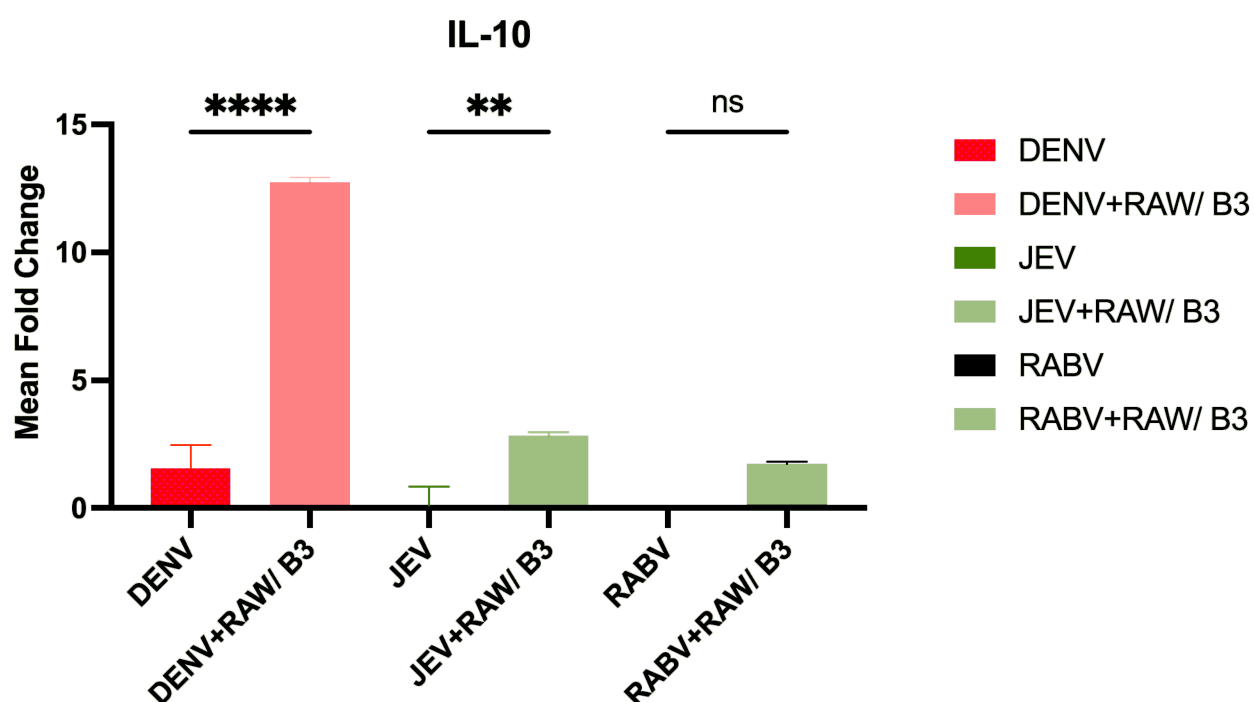


Fig 13 Expression of IL-10 in the bEnd3 cells post-exposure to different viruses. The results reflect the means $\pm$ SEMs from three different replicates. (Asterisks represent significant differences, compared to control.; ns, not significant * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$.)

Table 18 Gene expression of IL-10 after virus exposure to bEnd3 cells

Group	Mean Fold Change
DENV	1.54
DENV+RAW/B3	12.74
JEV	0.12
JEV+RAW/B3	2.83
RABV	0.03
RABV+RAW/B3	1.73

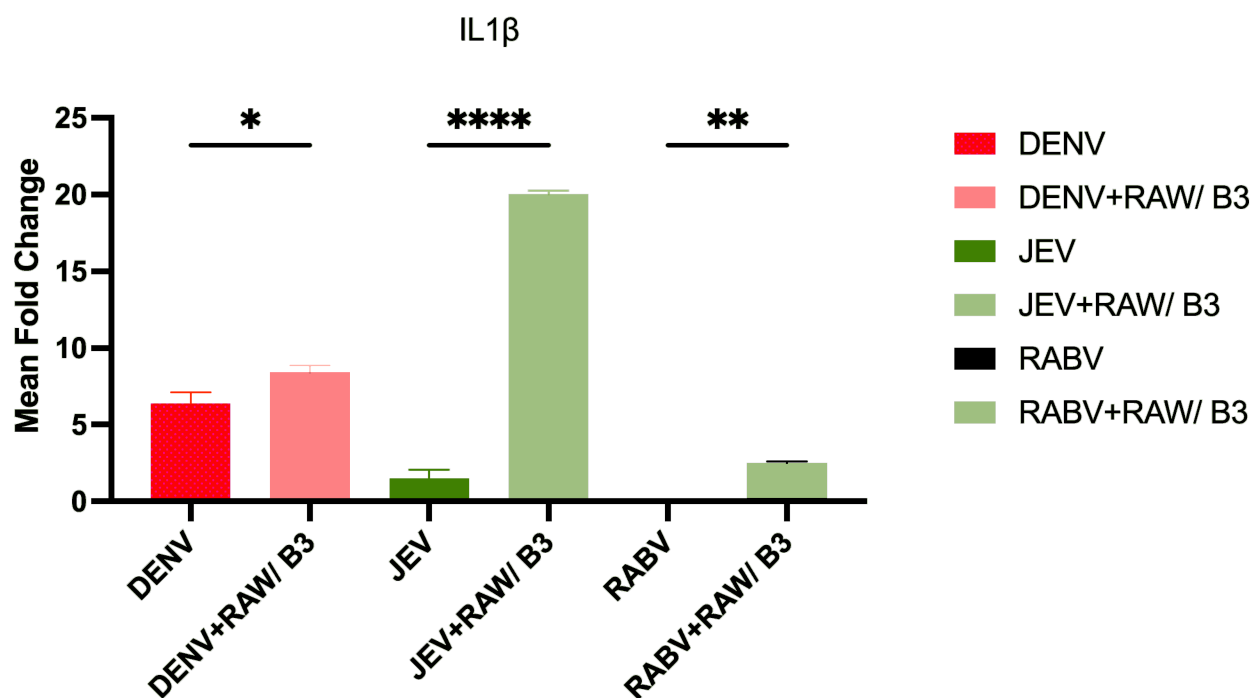


Fig 14 Expression of IL-1beta in the bEnd3 cells post-exposure to different viruses. The results reflect the means $\pm$ SEMs from three different replicates. (Asterisks represent significant differences, compared to control.; ns, not significant * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$.)

Table 19 Gene expression of IL-1beta after virus exposure to bEnd3 cells

Group	Mean Fold Change
DENV	6.36
DENV+RAW/B3	8.4
JEV	1.51
JEV+RAW/B3	20.06
RABV	0.04
RABV+RAW/B3	2.52

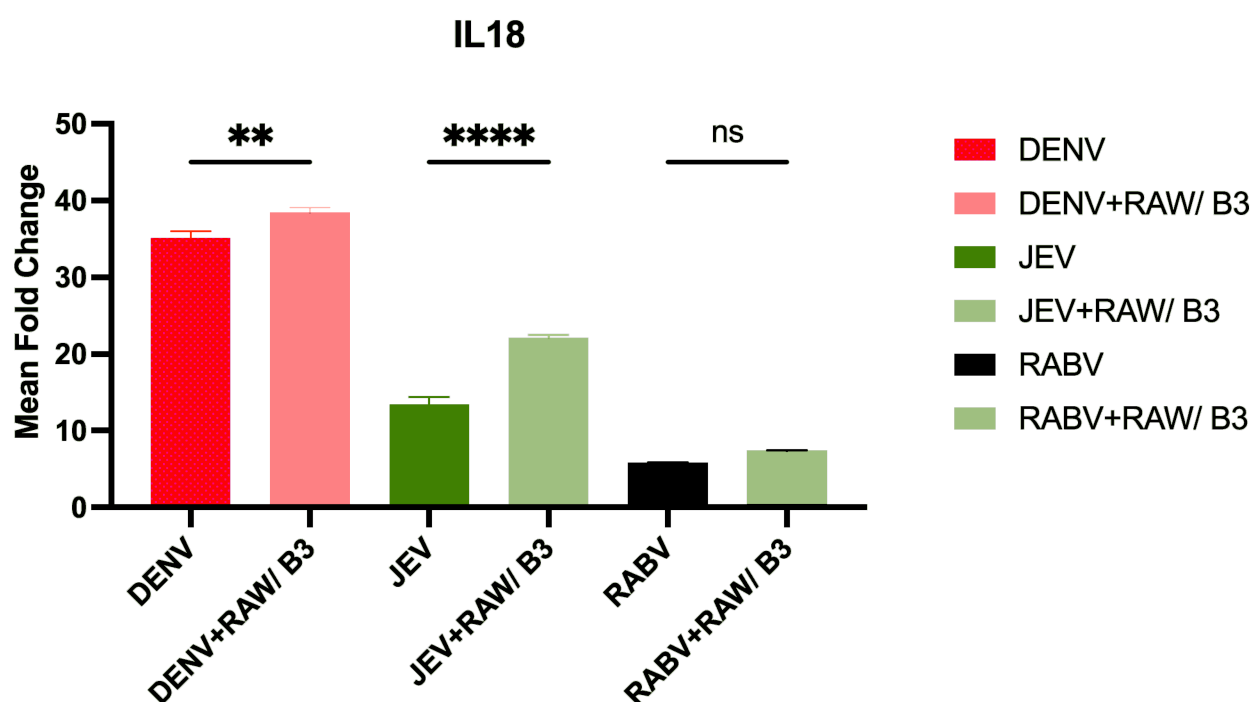


Fig 15 Expression of IL-18 in the bEnd3 cells post-exposure to different viruses. The results reflect the means $\pm$ SEMs from three different replicates. (Asterisks represent significant differences, compared to control.; ns, not significant * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$.)

Table 20 Gene expression of IL-18 after virus exposure to bEnd3 cells

Group	Mean Fold Change
DENV	35.09839935
DENV+RAW/B3	38.42
JEV	13.45434264
JEV+RAW/B3	22.11536
RABV	5.809175169
RABV+RAW/B3	7.41261

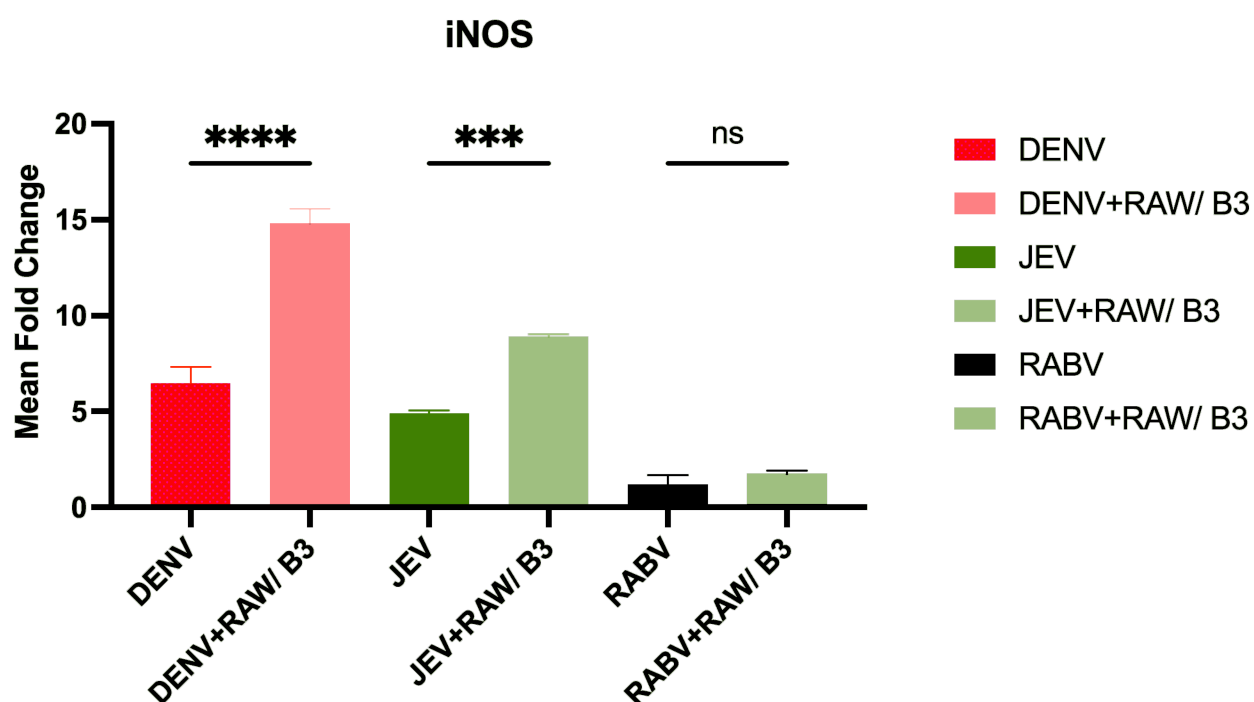


Fig 16 Expression of iNOS in the bEnd3 cells post-exposure to different viruses. The results reflect the means $\pm$ SEMs from three different replicates. (Asterisks represent significant differences, compared to control.; ns, not significant * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$.)

Table 21 Gene expression of iNOS after virus exposure to bEnd3 cells

Group	Mean Fold Change
DENV	6.46
DENV+RAW/B3	14.8
JEV	4.95
JEV+RAW/B3	8.89
RABV	1.20
RABV+RAW/B3	1.76

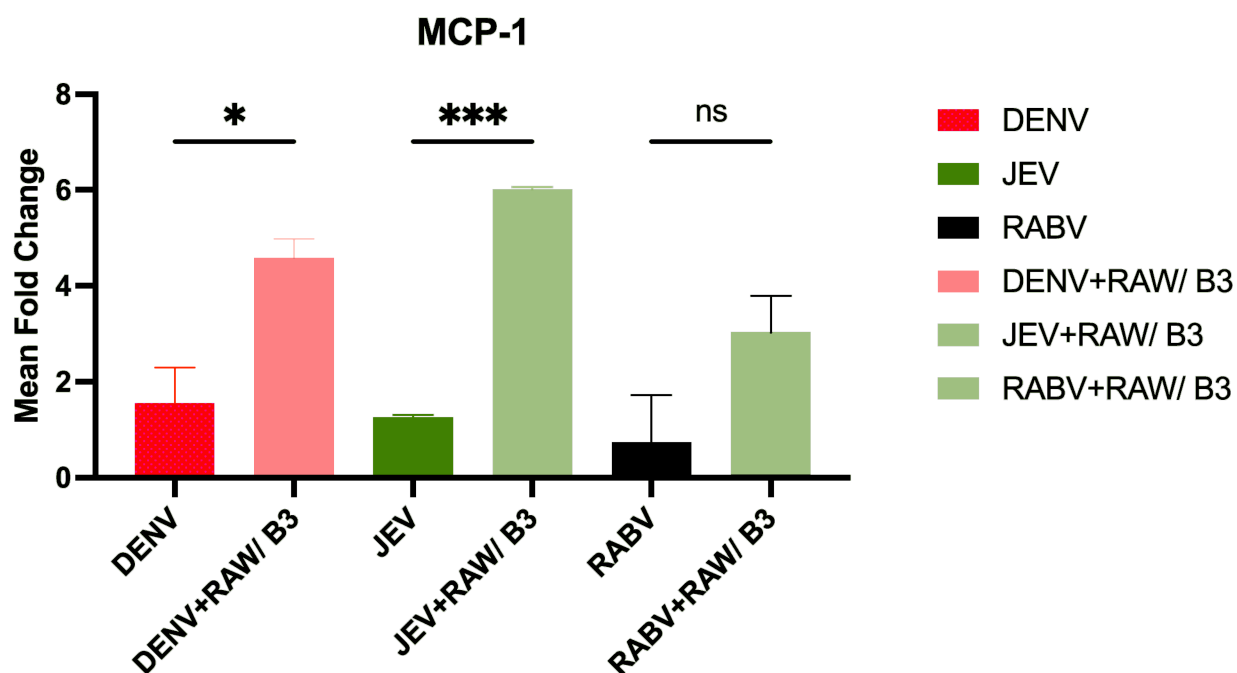


Fig 17 Expression of MCP-1 in the bEnd3 cells post-exposure to different viruses. The results reflect the means $\pm$ SEMs from three different replicates (Asterisks represent significant differences, compared to control.; ns, not significant * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$.)

Table 22 Gene expression of MCP-1 after virus exposure to bEnd3 cells

Group	Mean Fold Change
DENV	1.55
DENV+RAW/B3	4.59
JEV	1.26
JEV+RAW/B3	6.02
RABV	0.74
RABV+RAW/B3	3.03

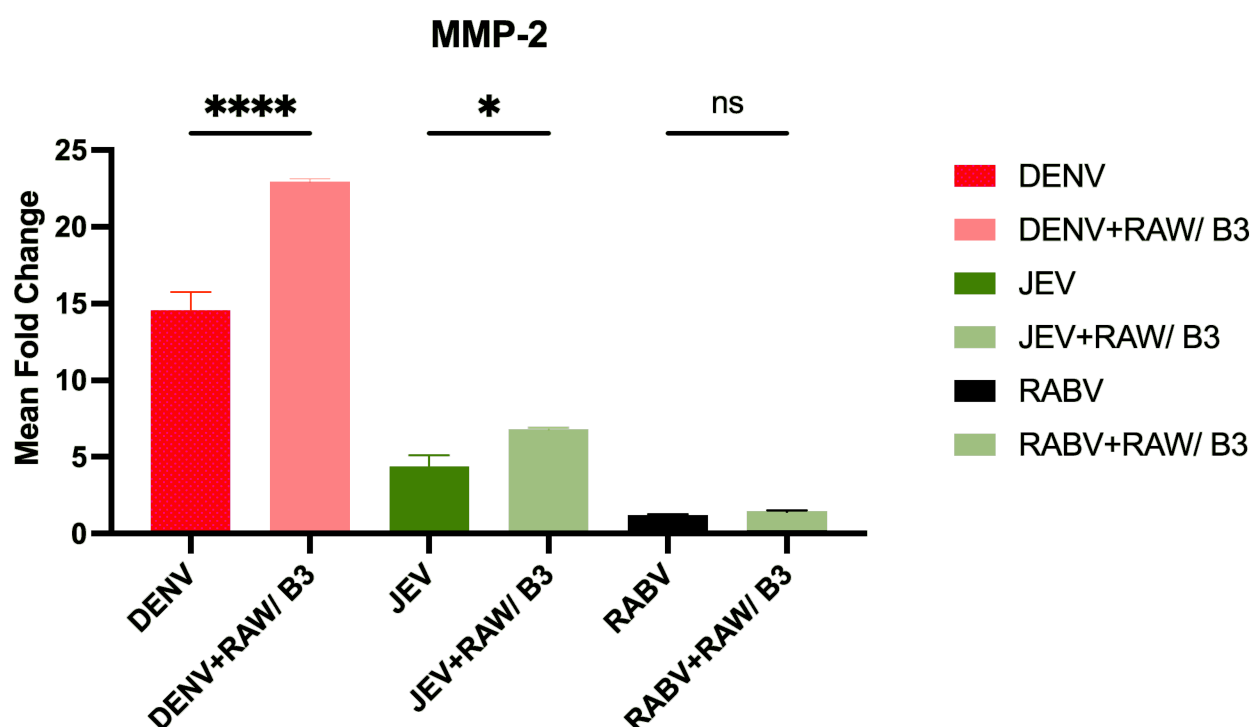


Fig 18 Expression of MMP-2 in the bEnd3 cells post-exposure to different viruses. The results reflect the means $\pm$ SEMs from three different replicates. (Asterisks represent significant differences, compared to control.; ns, not significant * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$.)

Table 23 Gene expression of MMP-2 after virus exposure to bEnd3 cells

Group	Mean Fold Change
DENV	14.55
DENV+RAW/B3	22.95
JEV	4.38
JEV+RAW/B3	6.78
RABV	1.22
RABV+RAW/B3	1.45

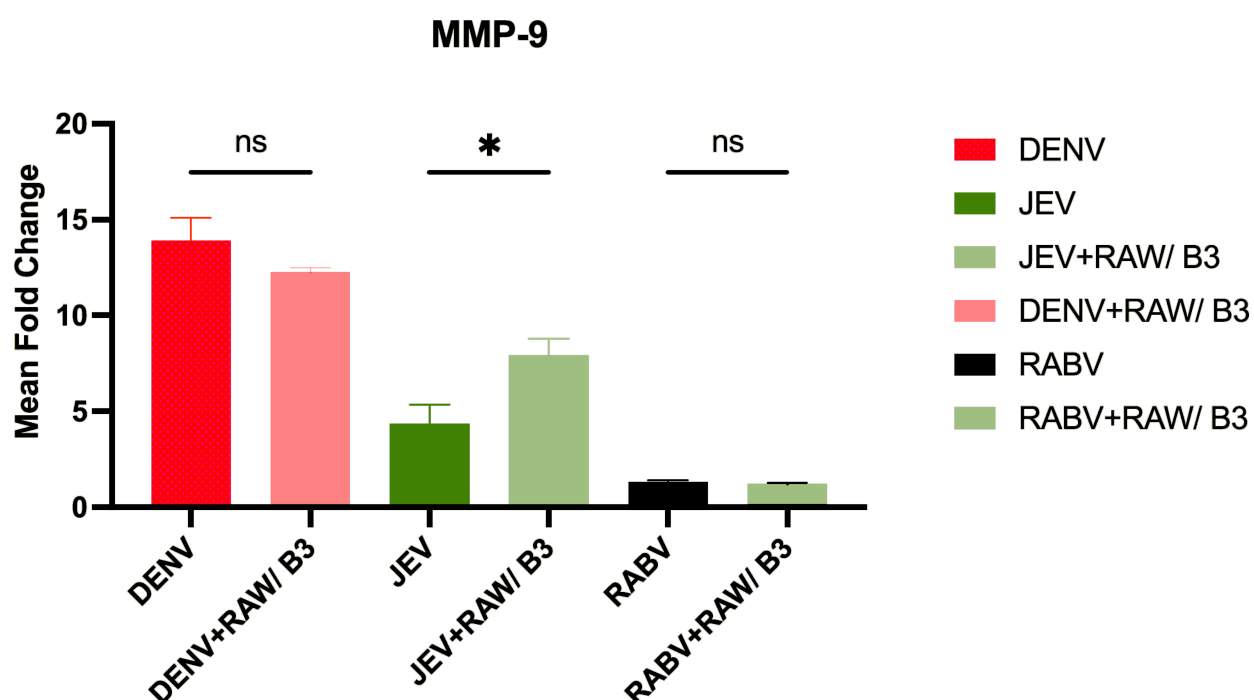


Fig 19 Expression of MMP-9 in the bEnd3 cells post-exposure to different viruses. The results reflect the means $\pm$ SEMs from three different replicates. (Asterisks represent significant differences, compared to control.; ns, not significant $p \leq 0.05$; $**p \leq 0.01$; $***p \leq 0.001$; $****p \leq 0.0001$.)

Table 24 Gene expression of MMP-9 after virus exposure to bEnd3 cells

Group	Mean Fold Change
DENV	13.89
DENV+RAW/B3	12.27
JEV	4.34
JEV+RAW/B3	7.92
RABV	1.32
RABV+RAW/B3	1.21

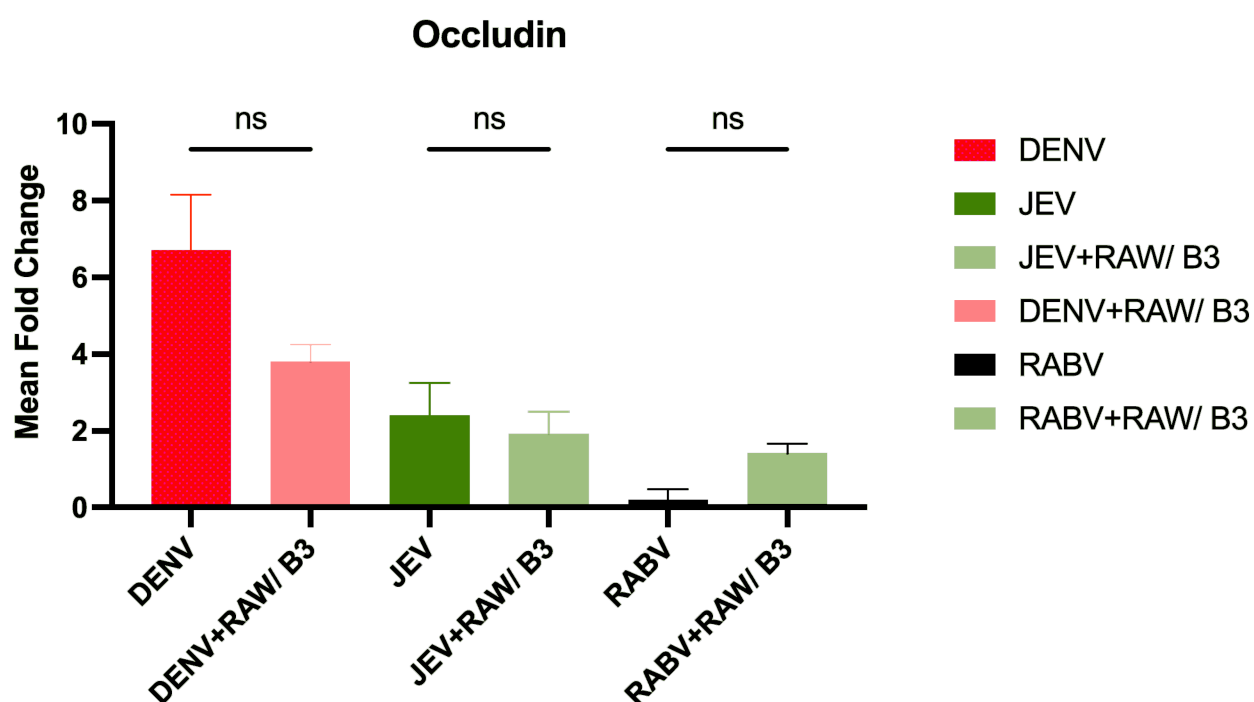


Fig 20 Expression of Occludin in the bEnd3 cells post-exposure to different viruses. The results reflect the means $\pm$ SEMs from three different replicates. (Asterisks represent significant differences, compared to control.; ns, not significant * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$).

Table 25 Gene expression of Occludin after virus exposure to bEnd3 cells

Group	Mean Fold Change
DENV	6.71
DENV+RAW/B3	3.8
JEV	2.39
JEV+RAW/B3	1.92
RABV	0.19
RABV+RAW/B3	1.42

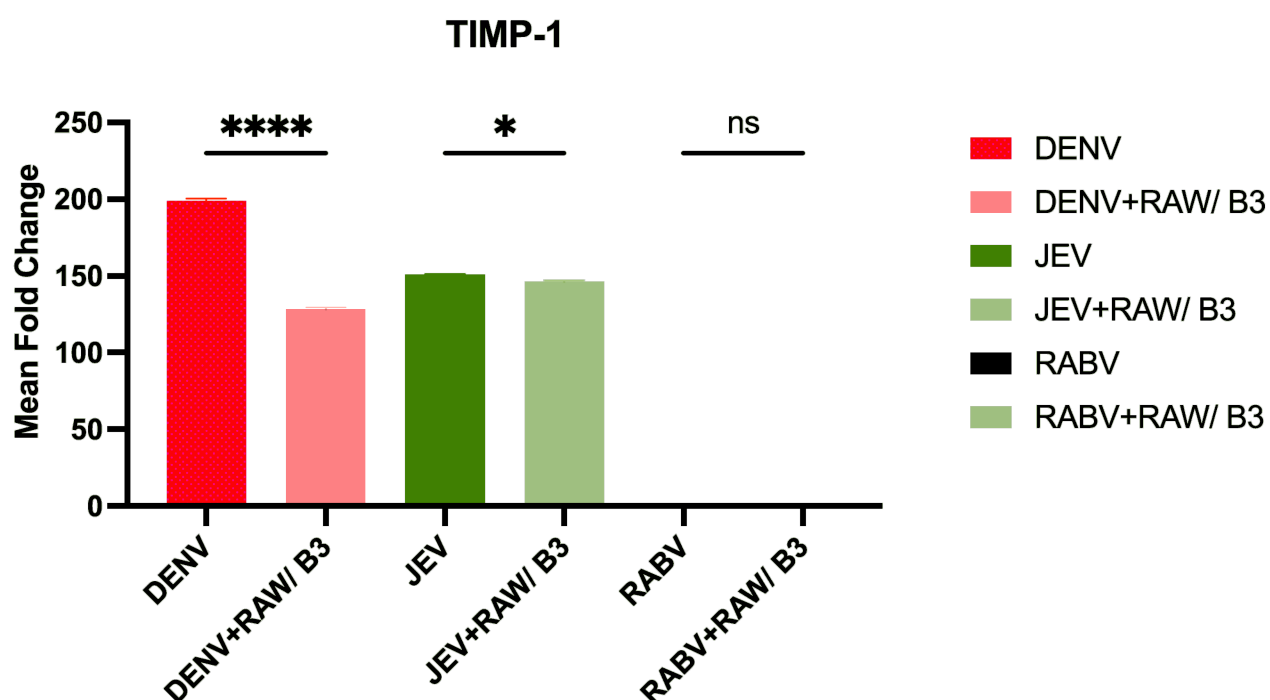


Fig 21 Expression of TIMP-1 in the bEnd3 cells post-exposure to different viruses. The results reflect the means $\pm$ SEMs from three different replicates. (Asterisks represent significant differences, compared to control.; ns, not significant * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$.)

Table 26 Gene expression of TIMP-1 after virus exposure to bEnd3 cells

Group	Mean Fold Change
DENV	198.85
DENV+RAW/B3	128.14
JEV	150.77
JEV+RAW/B3	146.13
RABV	0.008
RABV+RAW/B3	1.14

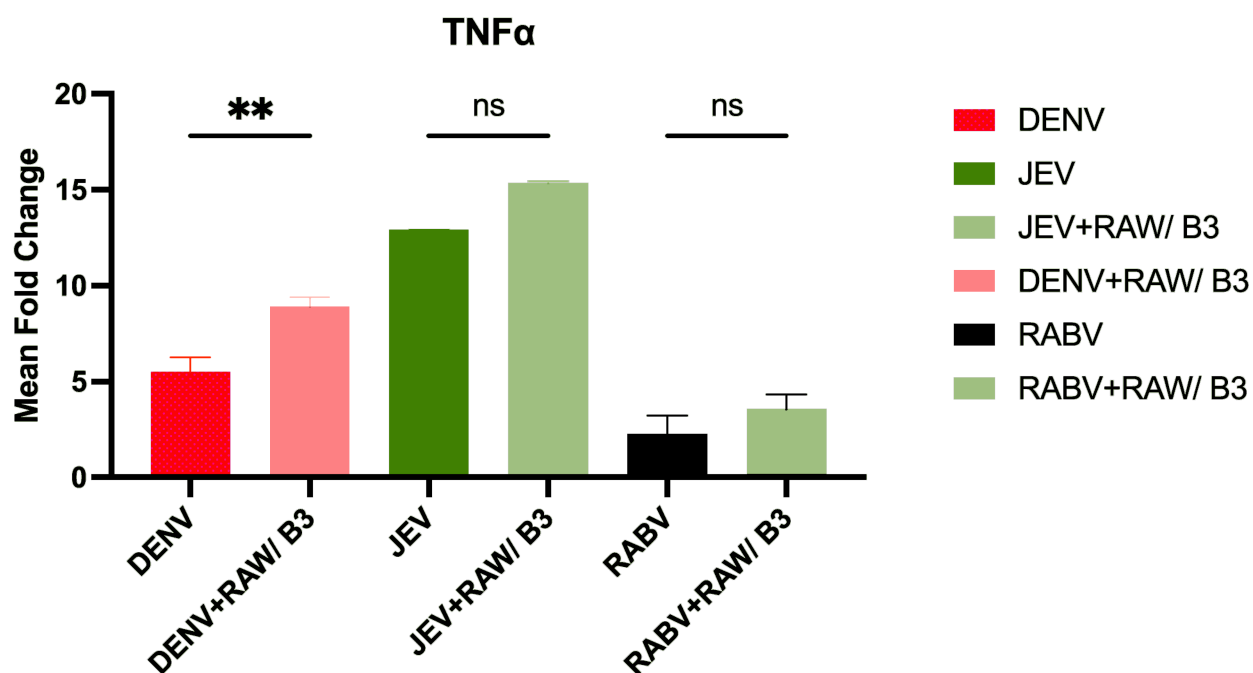


Fig 22 Expression of TNF-alpha in the bEnd3 cells post-exposure to different viruses. The results reflect the means $\pm$ SEMs from three different replicates. (Asterisks represent significant differences, compared to control.; ns, not significant * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$.)

Table 27 Gene expression of TNF-alpha after virus exposure to bEnd3 cells

Group	Mean Fold Change
DENV	5.51
DENV+RAW/B3	8.9
JEV	12.90
JEV+RAW/B3	15.35
RABV	2.25
RABV+RAW/B3	3.57

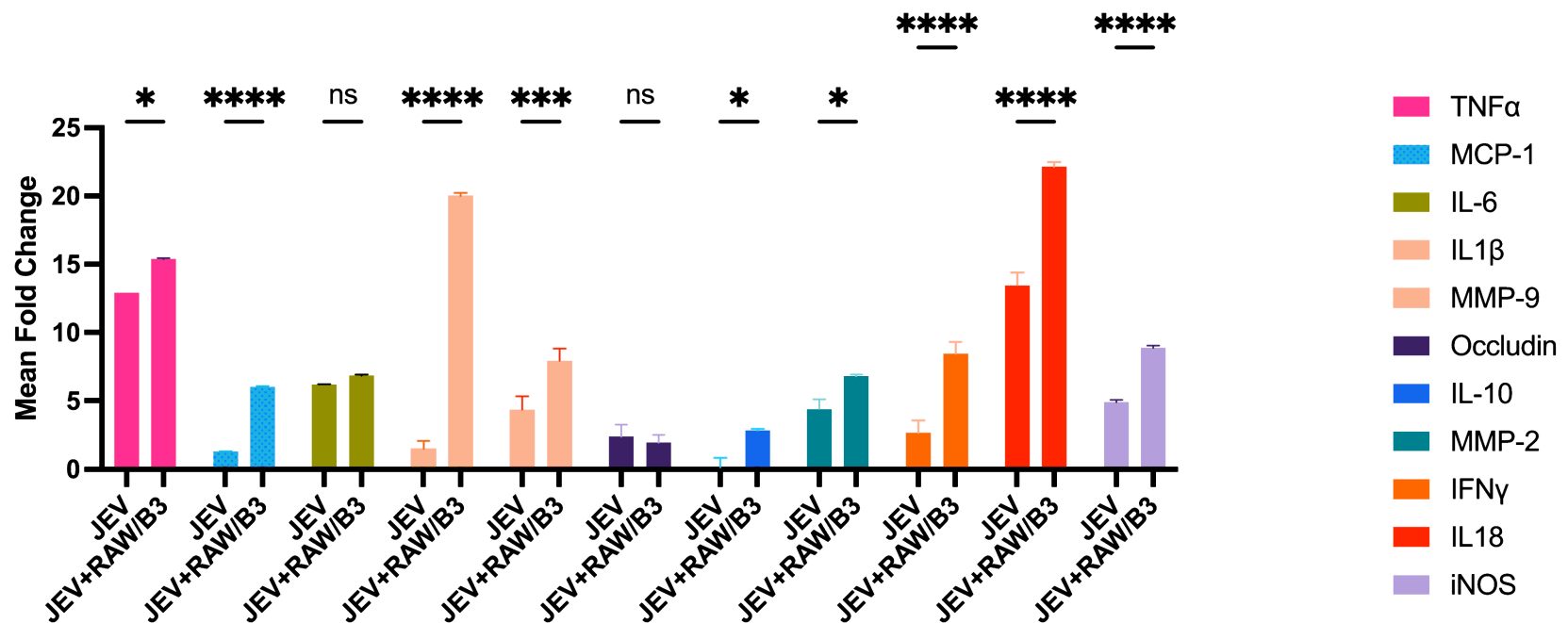


Fig 24 Expression profile of host factors in the bEnd3 cells post-exposure to Japanese encephalitis virus. The results reflect the means $\pm$ SEMs from three different replicates. (Asterisks represent significant differences, compared to control.; ns, not significant * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$.)

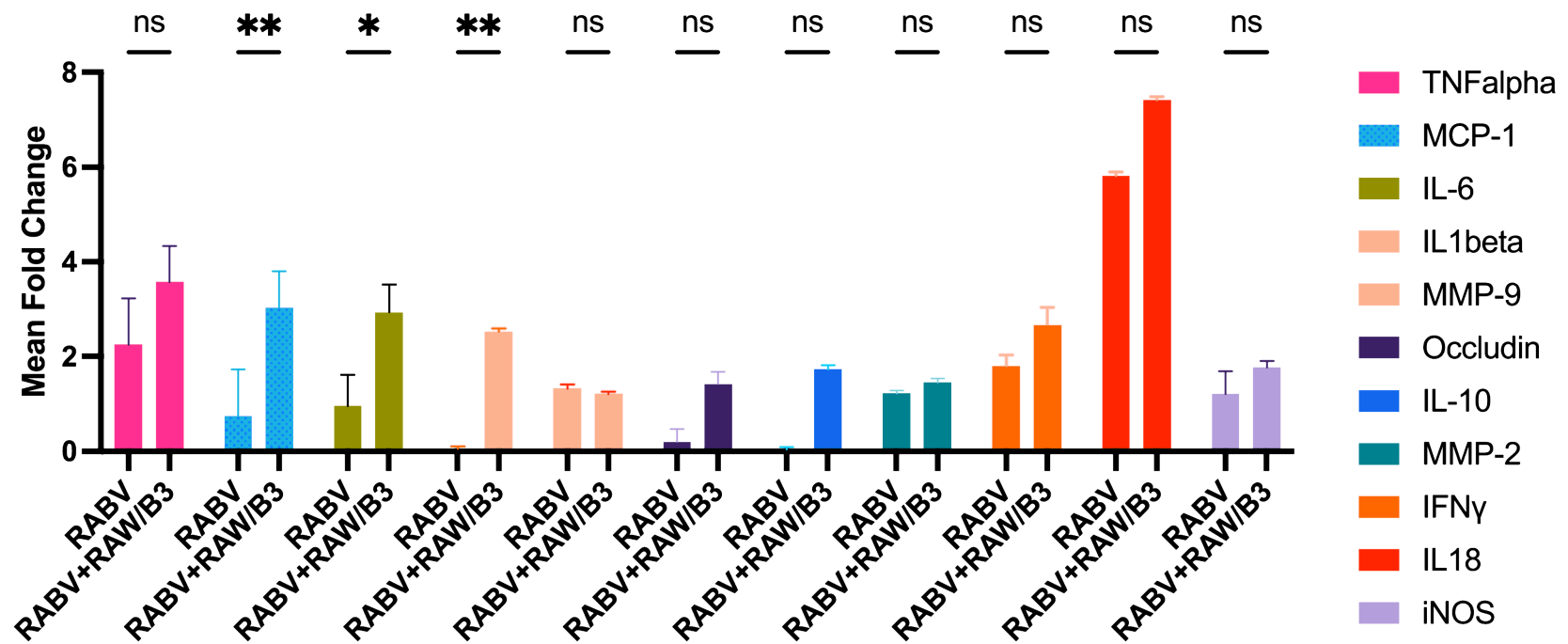


Fig 25 Expression profile of host factors in the bEnd3 cells post-exposure to rabies virus. The results reflect the means $\pm$ SEMs from three different replicates. (Asterisks represent significant differences, compared to control.; ns, not significant * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$.)

Evaluation of GAPDH as a housekeeping gene in Lipopolysaccharide-exposed BV2 cell

In order to assess the impact of LPS (lipopolysaccharide) exposure on cellular gene expression, we performed a series of experiments on BV2 cells, a commonly used model for studying microglial activation. One of the initial steps in these experiments was to determine an appropriate reference gene, or "housekeeping" gene, to normalize our data. To do this, we compared the expression of the gene encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in untreated and LPS-exposed cells after a 4 hour exposure period. By analyzing the Ct value, a measure of the amount of starting template in the PCR reaction, we observed a fold change of 1.03 between the two groups. This relatively small difference in GAPDH expression between the two groups indicates that it would be a suitable reference gene for normalizing our data. As a result, all further gene expression measurements were normalized to the respective GAPDH levels in order to accurately compare changes in expression between the untreated and LPS-exposed cells.

Analysis of pro-inflammatory, regulatory, and BBB breakdown markers

In the present study, we exposed BV2 cells to LPS (100ng/mL) for a period of 4 hours in order to induce an inflammatory response. Our observations showed a shift in favor of pro-inflammatory markers, with the primary marker being TNF- α as shown in Table 1. However, it's worthy to note that this early response did not elicit any signs of oxidative stress related apoptosis, as seen by an insignificant rise in the mRNA levels of iNOS. This led us to investigate downstream markers in order to better understand the specific changes that were occurring in the cells. Upon further examination, we observed a similar shift in the regulatory markers under investigation towards inflammation and chemotaxis dependent recruitment of neutrophils and other mononucleocytes as shown in Table 2. In particular, the increment in the expression of IL-6, which is a marker indicated for increased vascular permeability, from microglial cells led

us to assess the levels of gelatinases and occludin associated with the breakdown of the blood-brain barrier (BBB). In agreement with our hypothesis, we indeed observed the markers to be regulated in favor of this compromise. Though we had expected both gelatinases under evaluation (MMP-2 and MMP-9) to increase, we did not observe any significant rise in the MMP-9 mRNA levels as shown in Table 3. We presume that our analysis period of 4 hours was insufficient to detect this increase, since its inhibitor TIMP-1 had already started being down-regulated due to LPS induction. This suggests that a longer period of LPS exposure may be necessary in order to fully observe the effects of MMP-9 on BBB breakdown.

Target gene	ΔCt Base line (Mean $\pm$ SD)	ΔCt LPS (Mean $\pm$ SD)	Fold Change
IL-1 β	10.69 $\pm$ 0.89	9.34 $\pm$ 1.66	2.57
TNF- α	5.93 $\pm$ 1.29	3.88 $\pm$ 1.64	4.16
IL-10	10.23 $\pm$ 0.22	11.92 $\pm$ 0.43	-3.24
IFN- γ	8.33 $\pm$ 0.80	11.91 $\pm$ 0.87	-12.00
iNOS	7.84 $\pm$ 0.71	7.74 $\pm$ 0.96	1.07
IL-18	12.60 $\pm$ 0.59	12.26 $\pm$ 0.41	1.25

Table 25 Gene expression of cytokines involved in inducing inflammation after LPS exposure for 4 hours

Target gene	ΔCt Base line (Mean ± SD)	ΔCt LPS (Mean ± SD)	Fold Change
IL-6	11.35±1.43	10.19±0.97	2.23
MCP-1	5.40±0.55	4.70±0.45	1.62

Table 26 Gene expression of chemokines involved in regulating inflammation after LPS induction for 4 hours

Target gene	ΔCt Base line (Mean ± SD)	ΔCt LPS (Mean ± SD)	Fold Change
Occludin	8.28±0.64	11.53±0.45	-9.55
MMP-2	15.42±0.54	12.39±0.80	8.13
MMP-9	12.58±0.76	13.42±1.32	-1.79
TIMP-1	11.81±0.21	15.45±0.71	-12.49

Table 27 Gene expression of proteins involved in BBB compromise after LPS induction for 4 hours

4.5 Morphological changes on cells due to virus infection bEnd3 cells

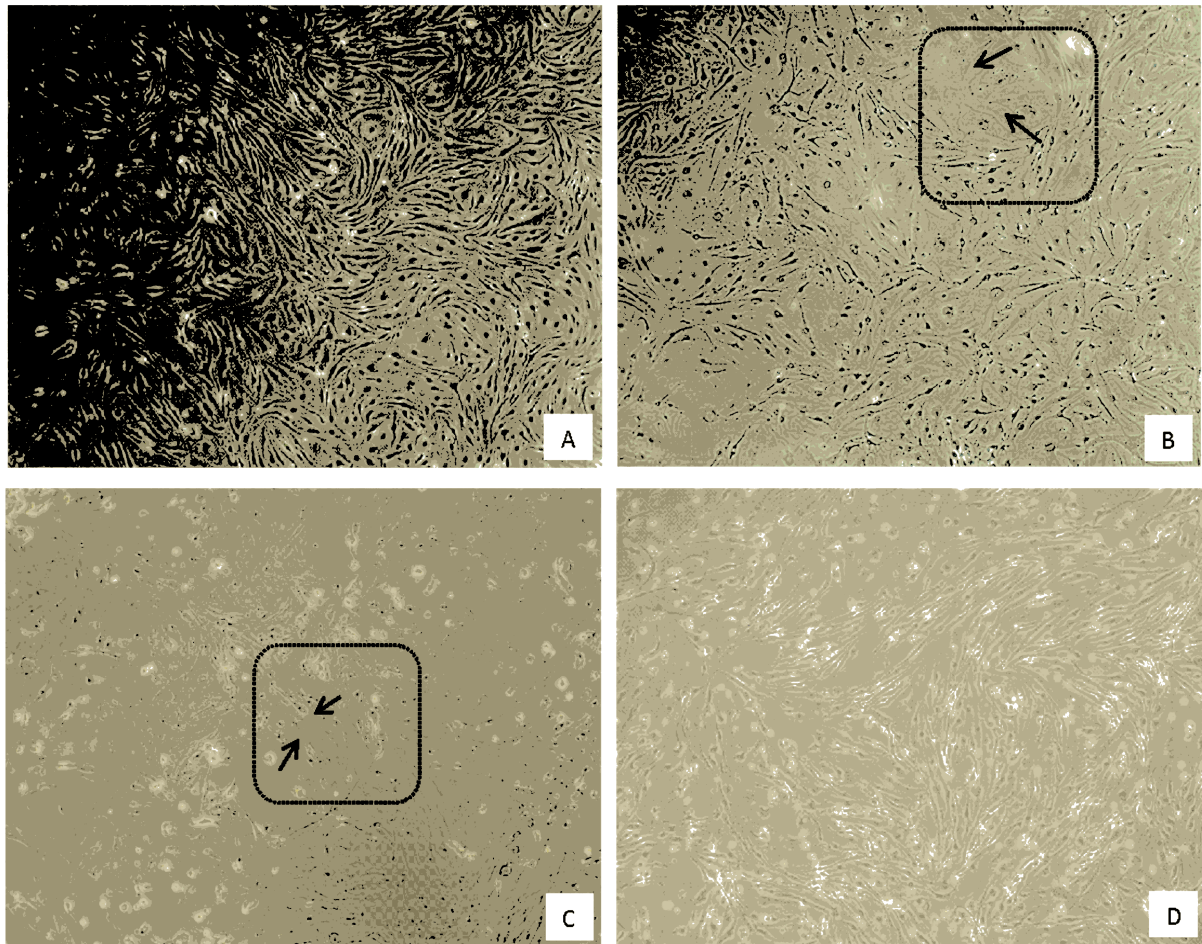


Fig 26 Effect on murine brain endothelial cells (bEnd3) when exposed with virus activated murine monocyte cells (RAW) A. bEnd3 Cell control, B. Loss of tight junction protein (indicated through arrows) indicating a clear BBB disruption caused due to DENV2 exposed to RAW supernatant infection, C. As compared to DENV2/+RAW infection, the (JEV GP-78/+RAW) has shown limited disruption of BBB tight junction caused due to JEV exposed to RAW supernatant infection, D. With rabies virus exposed to RAW monocytes (CVS/+RAW) has shown no significant change.

In this study, we examine the morphological changes that occur on murine brain endothelial cells (bEnd3) post virus exposure. Fig 25 illustrates the effect on bEnd3 cells with virus-activated murine monocyte cells (RAW). The first panel shows the bEnd3 cell control, while the second panel highlights the loss of tight junction protein, indicated by arrows, which suggests a clear disruption of the blood-brain barrier (BBB) caused by exposure to supernatants of DENV2 exposed murine monocytes (DENV2/+RAW). In contrast, the third panel shows

that JEV GP-78 has a limited disruption of the BBB tight junction compared to DENV2/+RAW infection. Finally, the fourth panel reveals that with supernatants of virus-exposed murine monocytic cells infection with rabies virus activated RAW monocytes (CVS/+RAW) has no significant change.

4.6 Transgenic lines crosses and breeding *Drosophila melanogaster*:

Table 28 Setup of *Drosophila* Fly Cross for glial disruption studies

UAS-NOS-RNAi (low expressed iNOS)		UAS-NOS; UAS-NOS (moderately expressed iNOS)		UAS-mac-NOS (super expressed iNOS)	
Moody-Gal4	Balanced with UAS- GFP 25 °C;18 °C	moody-Gal4	25 °C;18 °C	moody-Gal4	25 °C;18 °C
UAS-GFP; moody-Gal4	25 °C;18 °C	UAS-GFP; moody-Gal4	25 °C;18 °C	UAS-GFP; moody-Gal4	25 °C;18 °C
NP6293, UAS-GFP	25 °C;18 °C	NP6293, UAS-GFP	25 °C;18 °C	NP6293, UAS-GFP	No Correct Progeny
repo4.3- Gal4(II)	Balanced with UAS- GFP 25 °C;18 °C	repo4.3- Gal4(II)	No Correct Progeny	repo4.3- Gal4(II)	No Correct Progeny
repo-Gal4 (III)	Balanced with UAS- GFP 25 °C;18 °C	repo-Gal4 (III)	No Correct Progeny	repo-Gal4 (III)	No Correct Progeny

We conducted fly crosses using three different UAS-NOS-RNAi lines for iNOS (inducible Nitric Oxide Synthase) with varying expression levels (low, moderately, and super expressed iNOS). The fly crosses were performed with the moody-Gal4 driver line at both 25°C and 18°C temperatures. When crossed with the moody-Gal4 driver line at 25°C and 18°C, the UAS-NOS-RNAi lines for iNOS showed varying expression levels of GFP (Green Fluorescent Protein). The NP6293 UAS-GFP line was crossed with moody-Gal4 driver lines at 25°C and 18°C, but no correct progeny was obtained. The repo4.3-Gal4 (II) and repo-Gal4 (III) driver lines were crossed with UAS-GFP lines at 25°C, but no correct progeny was obtained. We found that the UAS-mac-NOS lines did not produce any correct marked progeny when crossed at 25°C. However, when crossed at 18°C, they were able to obtain correct transgenic progeny from some of the crosses. Before proceeding with the crosses involving reporter lines, the researchers first crossed the unbalanced fly lines with GFP, Pr, Tm6B, and Sb markers flies. The ideal breeding temperature for flies was determined to be 25°C. However, due to the lack of correct progeny from the UAS-mac-NOS lines at this temperature, the researchers successfully obtained correct transgenic progeny by breeding them at 18°C for some of the crosses. The results suggest that the expression of iNOS and the successful production of transgenic progeny in *Drosophila melanogaster* are influenced by the specific UAS-NOS-RNAi lines used and the temperature at which the crosses are performed.

4.7 Immunostaining with Anti-Alexa488 and Anti-Cy3 to analyze BBB integrity

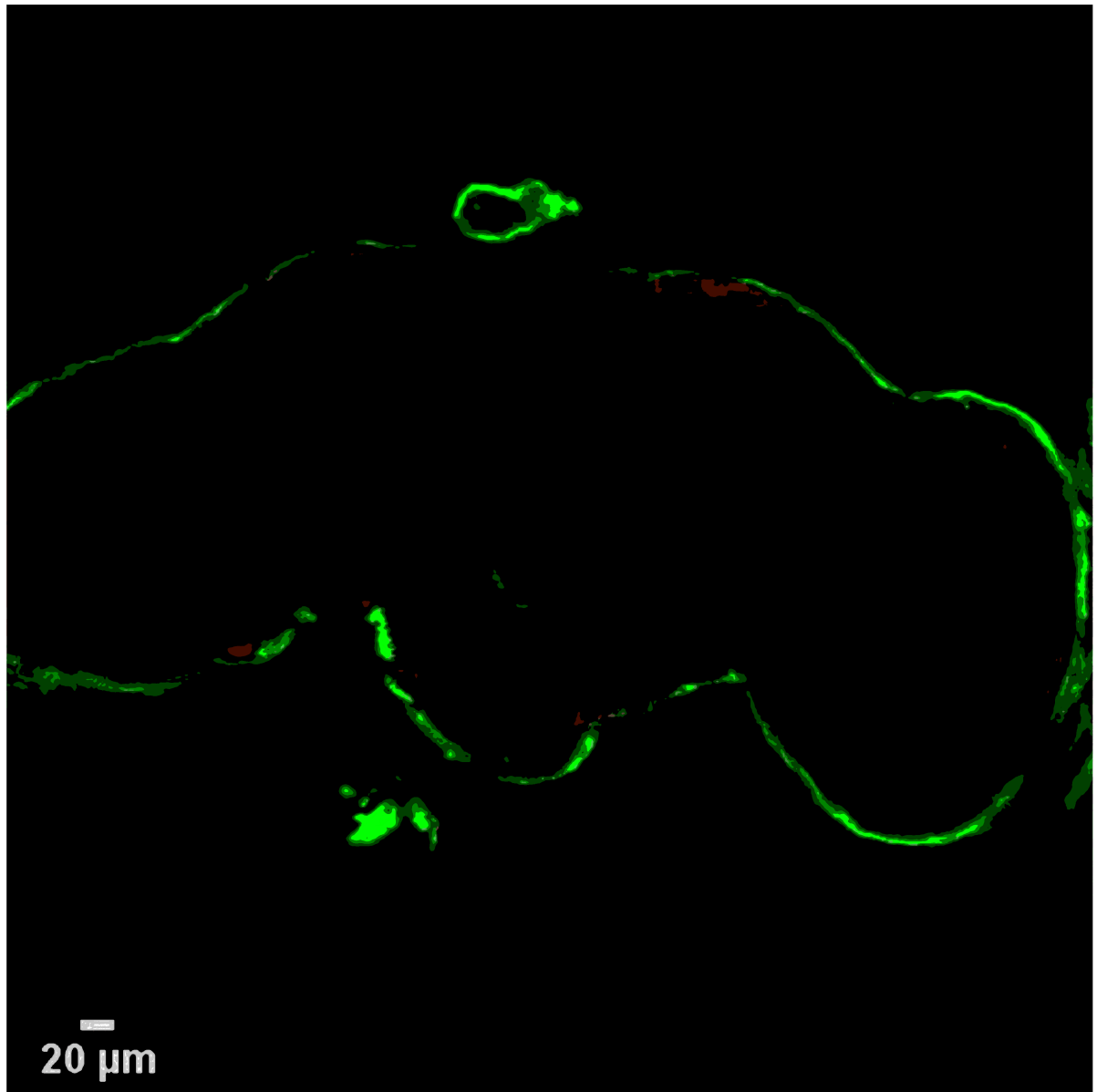


Fig 27 Confocal image of the wild type of Moody-UAS-GFP parent adult brain where the green fluorescence intact membrane lining indicates the presences of moody protein in the subperineurial glia barrier membrane. Whereas the red region confirms the presence of Brp protein which is ubiquitously expressed in *Drosophila* brain (Knapek, Stephan et al., 2009).

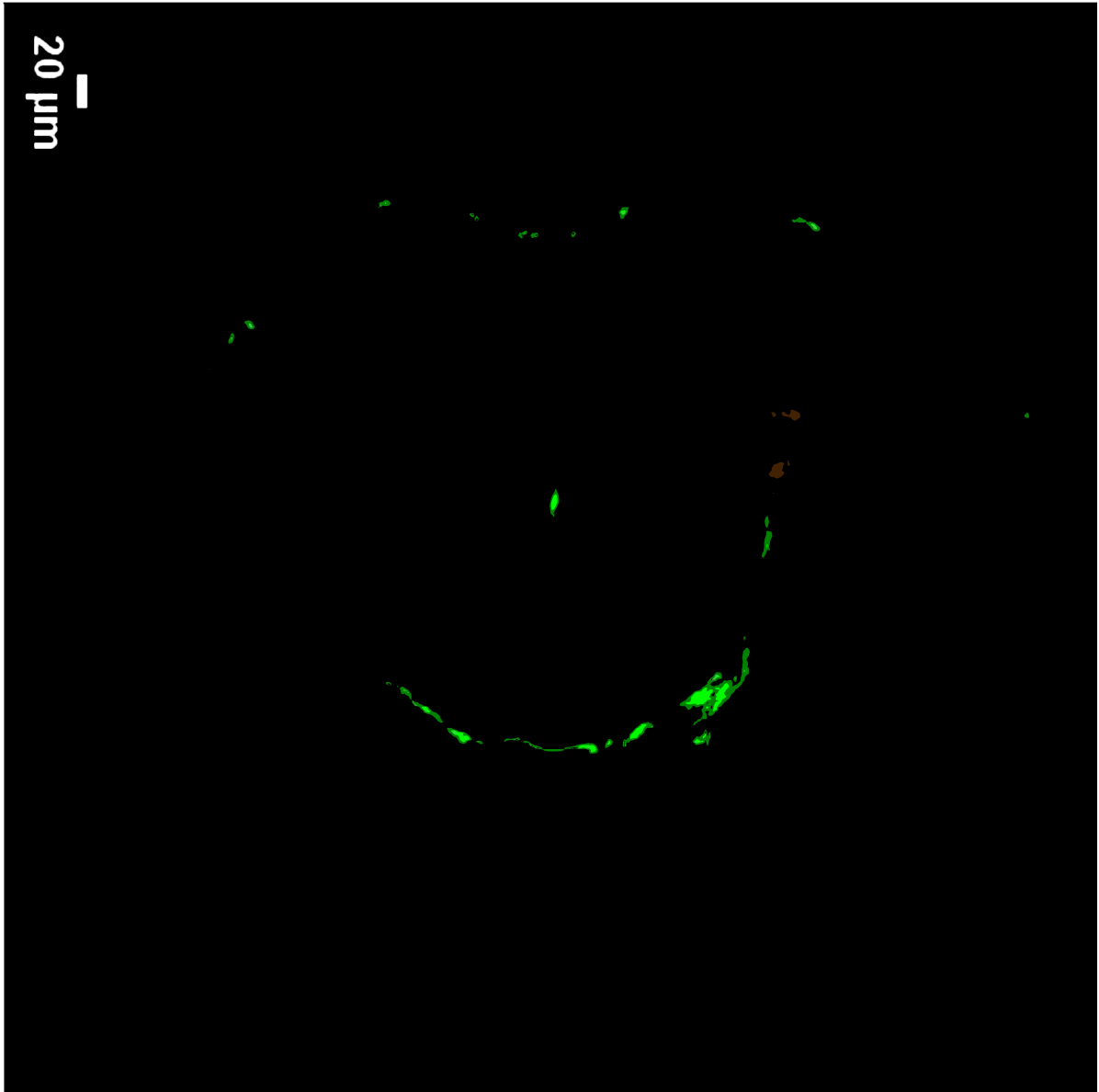


Fig 28 Effect of iNOS on Moody-UAS-GFP fly transgenic crosses with NOS-RNAi lines, the green fluorescence of moody protein could be partially observed across the glial barrier membrane. Also, the Brp signals could be observed in the whole *Drosophila* brain confocal section.

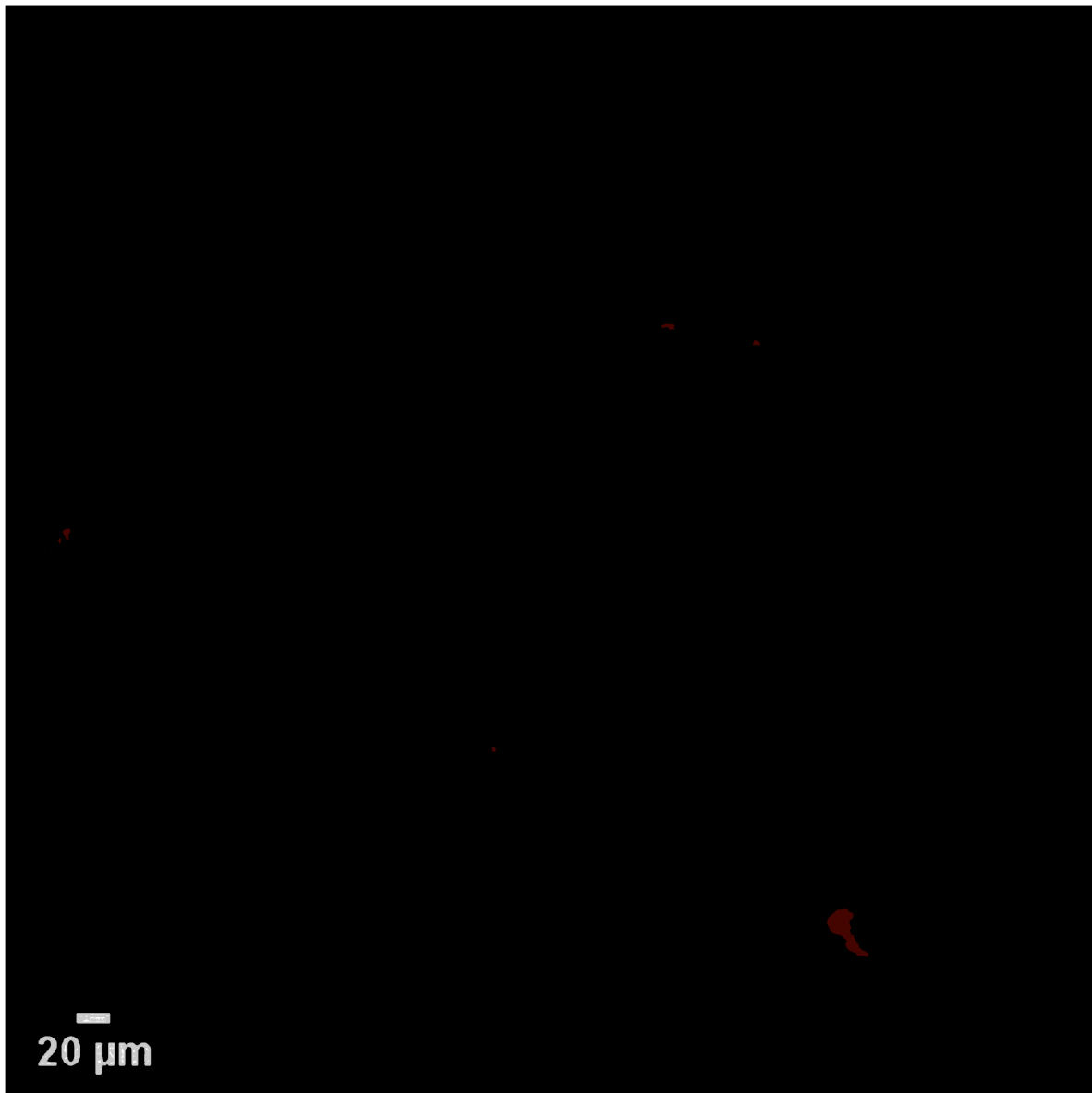


Fig 29 Effect of iNOS on Moody-UAS-GFP fly transgenic crosses with UAS-mac-NOS.

We can observed absence of green fluorescence lining indicating the loss of moody protein from the glial barrier membrane. The Brp (Bruch pilot, German for crash pilot, Wagh et al., 2006) protein signal could be observed in the whole *Drosophila* brain. All the above images were taken using Leica Airy Scan 800 with Z-tack image processing for Ch 1(Alexa488 Green) and Ch2 (Cy3 Red).

The results from immunostaining with Anti-Alexa488 and Anti-Cy3 to analyze blood-brain barrier (BBB) integrity in the transgenic crosses are presented in Fig 12. The control group,

represented by w-type Moody-UAS-GFP parent adult brain, showed normal BBB integrity. In the cross between Moody-UAS-GFP and UAS-mac-NOS lines, the BBB integrity was affected, as indicated by changes in the immunostaining pattern with Anti-Alexa488 and Anti-Cy3. The alterations observed in this cross suggest that the presence of UAS-mac-NOS may have an impact on BBB integrity. Conversely, in the cross between Moody-UAS-GFP and UAS-NOS-RNAi flies, the BBB integrity appeared to be preserved, as the immunostaining pattern was comparable to the control group. This suggests that the inhibition of iNOS using UAS-NOS-RNAi may have a protective effect on BBB integrity. The images were captured using Leica Airy Scan 800 with Z-stack image processing, allowing visualization of the BBB integrity through specific markers (Ch1: Alexa488 Green and Ch2: Cy3 Red). These findings indicate that iNOS plays a role in modulating BBB integrity in the Moody-UAS-GFP fly transgenic crosses. The presence of UAS-mac-NOS seems to disrupt BBB integrity, while inhibition of iNOS using UAS-NOS-RNAi appears to preserve it.

4.8 Disrupted glial brain barrier of *Dm* through TEM

The results of the study involving the analysis of the glial brain barrier in *Drosophila melanogaster* (Dm) through TEM (Transmission Electron Microscopy) are presented in Fig 13. We performed gene expression studies by dissecting 50-55 fly heads for each correct progeny of the transgenic lines. The dissected heads were immediately processed for RNA extraction using the NEB Monarch RNA isolation kit. For confocal imaging and electron microscopy, 10 adult fly brains were dissected for each study group cross. The brains were embedded in epoxy resin for TEM imaging. Clockwise from the top left, the TEM images show the following: Moody wild type adult brain embedded in epoxy resin, displaying an intact and smooth lining of the blood-brain barrier (BBB), Magnified view of the parent moody BBB, showing a preserved smooth lining in UAS-NOS-RNAi BBB lining, indicating the effect of inhibiting

iNOS on the BBB lining, Moody-UAS-mac-NOS adult brain with a damaged BBB membrane lining, indicating the impact of the presence of UAS-mac-NOS on the BBB. We initially followed David Bock et al.'s method for sample processing. However, we later changed the initial fixative to a mixture of fixative solution and attempted the High Pressure Freezing (HPF) technique to process a large number of samples. The TEM images were captured using the Hitachi H7600 TEM at 120 kV. Overall, the TEM images provide insights into the structural integrity of the glial brain barrier in *Drosophila melanogaster* under different conditions, including the effects of iNOS inhibition and the presence of UAS-mac-NOS. These findings suggest that iNOS and UAS-mac-NOS may play significant roles in modulating the integrity of the glial brain barrier in the studied transgenic crosses.

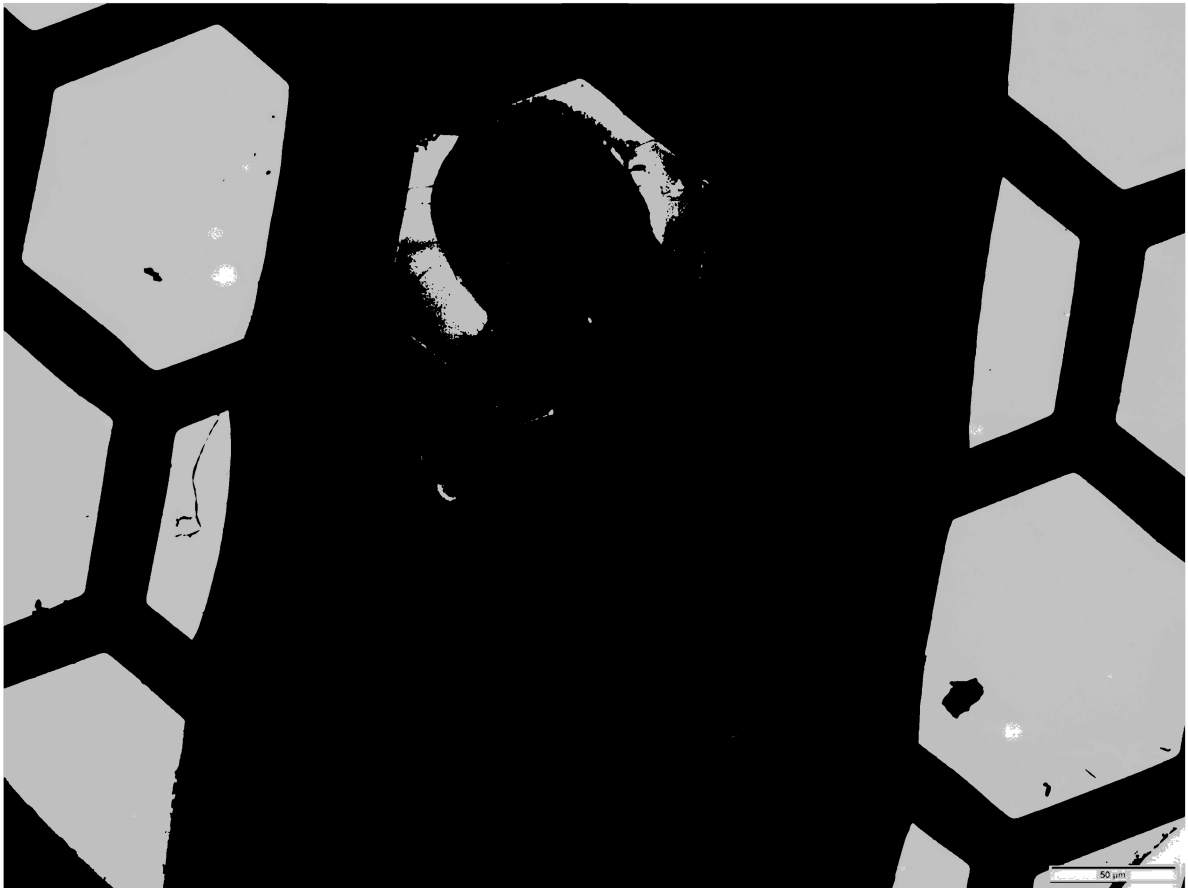


Fig 30 TEM images of parent moody wild type adult brain embedded in epoxy resin.

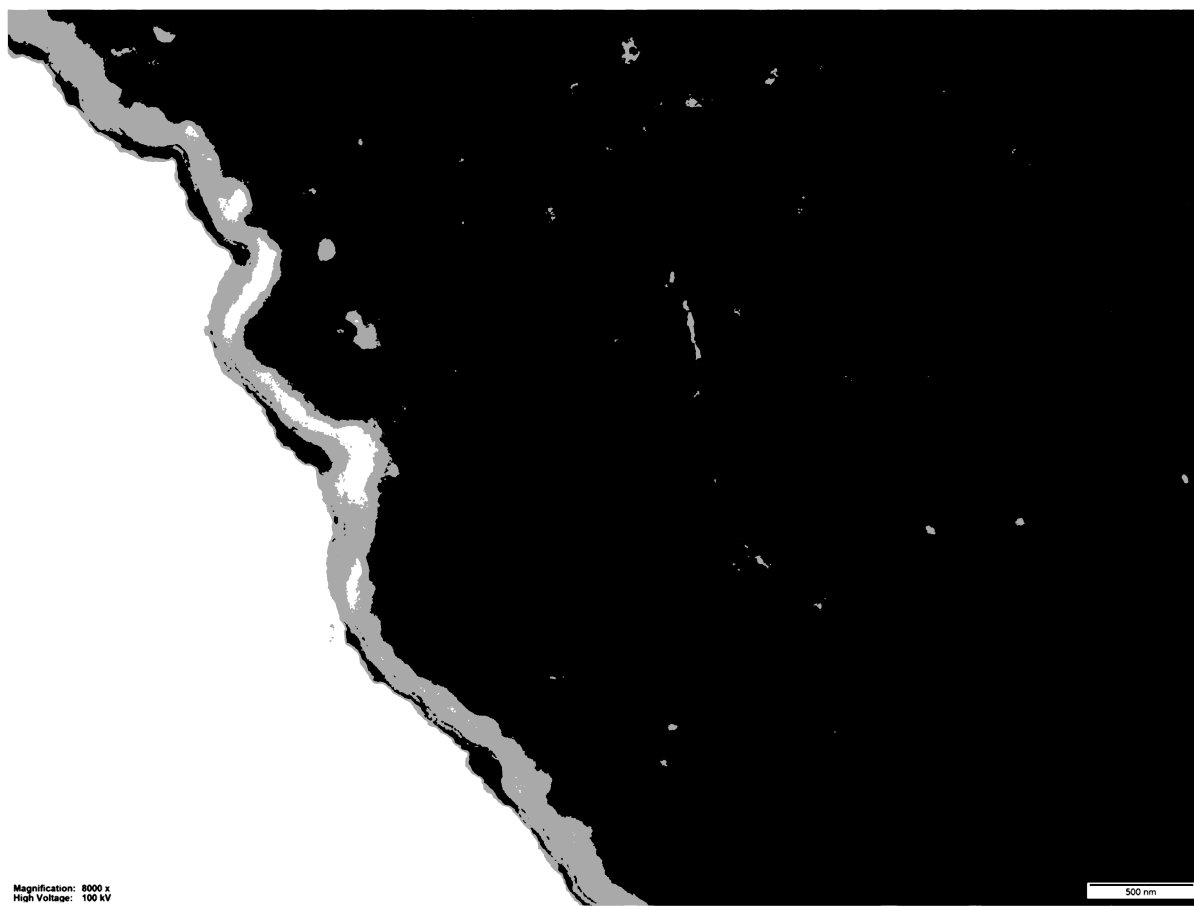


Fig 31 TEM images of moody w- type adult brain embedded in epoxy resin, magnified parent moody BBB with the intact smooth lining of glial barrier.



Fig 32 TEM images of moody w- type adult brain embedded in epoxy resin, Moody-UAS-NOS-RNAi adult brain focusing on the membrane lining. This image of glial membrane is consistent with the confocal image wherein the partial lining of the green moody lining could be observed across the glial membrane barrier.

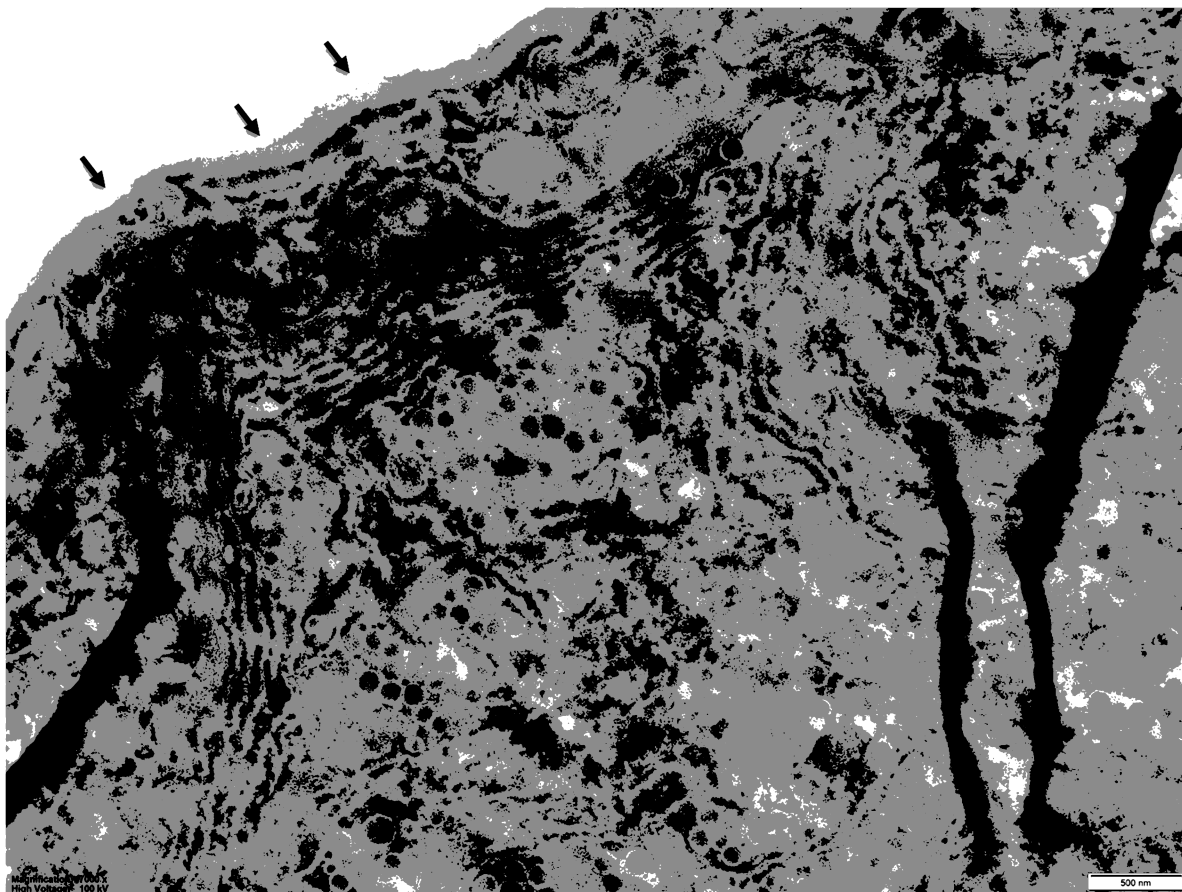


Fig 33 TEM images of Moody-UAS-mac-NOS adult brain focusing on the membrane lining (arrows showing the disrupted glial barrier).

The above images of moody wild type adult brain embedded in epoxy resin, magnified parent moody BBB intact smooth lining, UAS-NOS-RNAi BBB lining, and Moody-UAS-mac-NOS adult brain with damaged BBB membrane lining. The sample processing was initially followed with David Bock et.al method but then we changed the initial fixative with a mixture of fixative solution and tried High pressure freezing technique to process large number of samples. The images were captured using Hitachi H7600 TEM 120 kV.

4.9 Optimization of RNA extractions method for *Drosophila* fly brain

Table 29 *Drosophila* RNA quantified from different methods of RNA extraction.

Optimization for RNA extraction from fly brains	ng/ μ L
Qiagen (without Rnase Later)	2.6
Qiagen (with RNase Later)	3.0
NEB Monarch + Rnase Later	11.6
NEB Monarch + DNA/RNA Protect Reagent	10.1
NEB Monarch + DNA/RNA Protect Reagent + Bead Homogenization (3 mm)	3.7
NEB_Monarch + DNA/RNA Protect Reagent + Bead Homogenization (1.45 mm)	521.6



Fig 34 Expression of iNOS, dmMMP1, dmMMP2 and moody expression in wild type and NP-RNAi crossed flies on 2% agarose gel.

Based on the results, the New England Biolabs (NEB) Monarch + DNA/RNA Protect Reagent with Bead Homogenization using 1.45 mm beads resulted in the highest RNA yield, with a

concentration of 521.6 ng/ μ L. This method appears to be the most efficient for extracting total RNA from the samples tested.

4.10 Gene expression of glial barrier proteins of *Drosophila* fly

In this study, a comprehensive analysis was conducted utilizing 2-way ANOVA to assess the significant differences in gene expression. The results revealed highly significant differences with a p-value of <0.0001 . Specifically, the study focused on evaluating the marker proteins moody and nrx4 (neurexin IV or Nr_x4 or Nr_xIV) to assess the septate junction lining of the glial membrane. Notably, when UAS-mac-NOS flies were crossed with wild moody-GFP-Gal4 parents, a substantial increase in iNOS expression was observed, subsequently leading to a reduction in dmMMPs expression. Furthermore, this modulation was associated with a significant decrease in moody and nrx4 expression, particularly evident in the crosses involving moody. The findings depicted in the below graphs illustrate the expression patterns of iNOS, dmMMP1, dmMMP2, and moody in various fly lines crossed with NOS-RNAi and distinct NOS driver lines, shedding light on the intricate interplay and regulatory mechanisms underlying these critical genes.

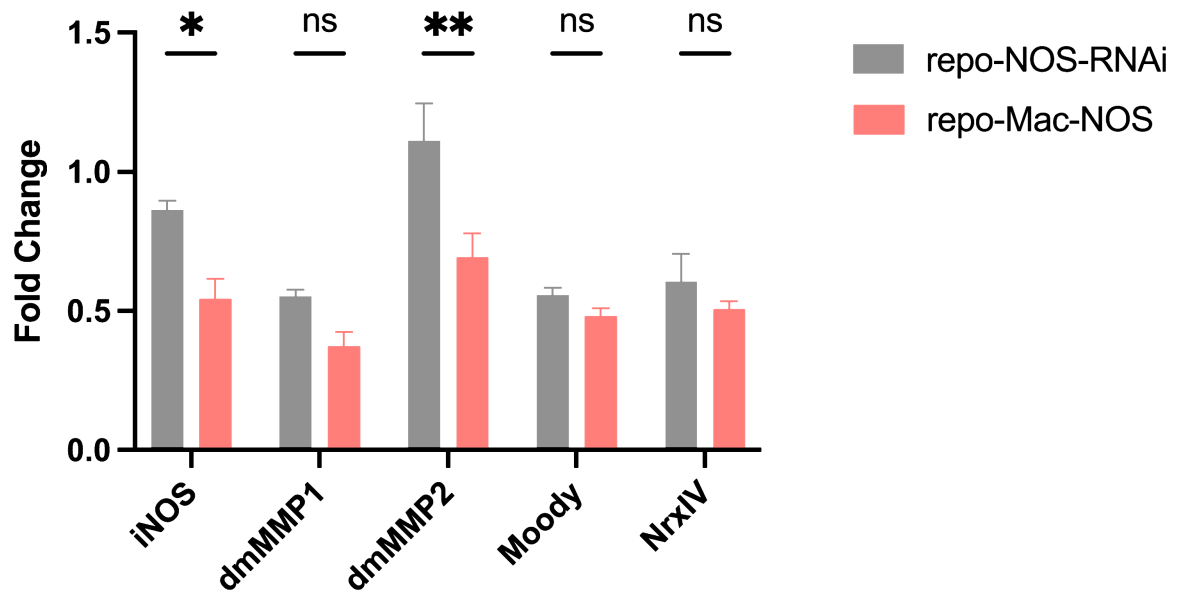


Fig 35 Expression of iNOS, dmMMP1, dmMMP2 and moody expression in wild type and repo lines flies crossed with RNAi and Mac-NOS driver lines flies. The results reflect the means $\pm$ SEMs from three different replicates. (Asterisks represent significant differences, compared to control.; ns, not significant * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$.)

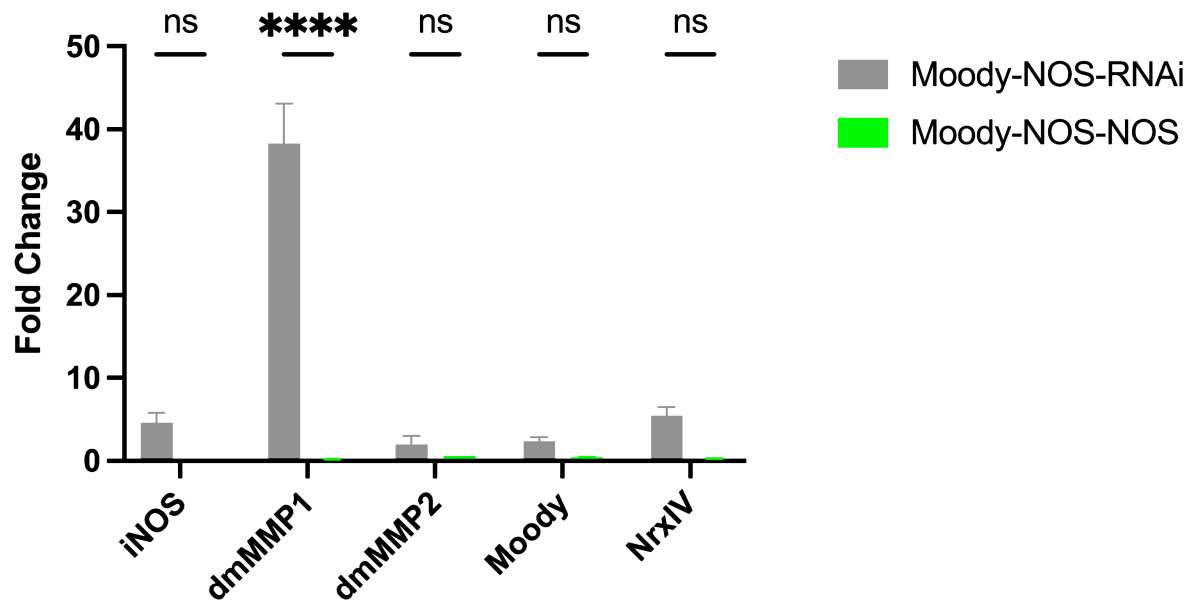


Fig 36 Expression of iNOS, dmMMP1, dmMMP2 and moody expression in wild type and Moody-GFP-Gal4 flies crossed with RNAi and NOS-NOS driver lines flies. The results reflect the means $\pm$ SEMs from three different replicates. (Asterisks represent significant differences, compared to control.; ns, not significant * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$.)

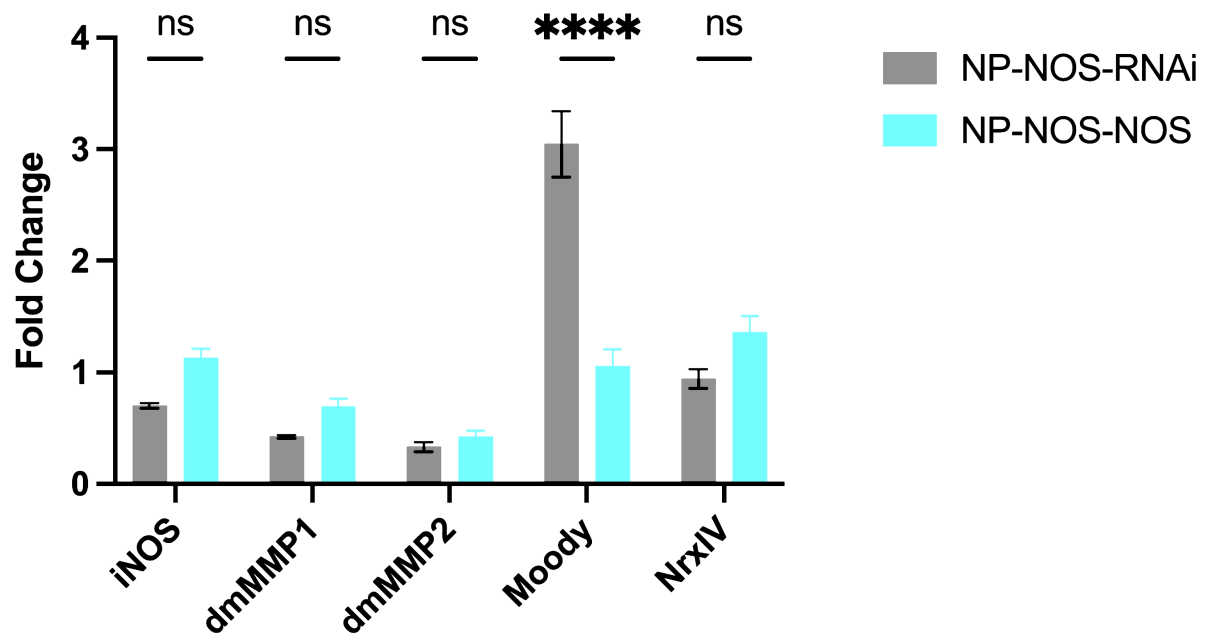


Fig 37 Expression of iNOS. dmMMP1, dmMMP2 and moody expression in wild type and NP6293-GFP flies crossed with RNAi and NOS-NOS driver lines flies. The results reflect the means $\pm$ SEMs from three different replicates. (Asterisks represent significant differences, compared to control.; ns, not significant * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$.)

Moody and NrxFV (neurexin IV) are used as marker proteins to evaluate the septate junction lining of the glial membrane. When UAS-mac-NOS flies are crossed with w- moody-GFP-Gal4 parent flies, there is an increase in iNOS expression. This increase in iNOS expression leads to a reduction in the expression of dmMMPs (*Drosophila* Matrix Metalloproteinases). As a result of the above-mentioned cross, there is a significant decrease in moody and NrxFV expression in moody crosses. This suggests that iNOS activity impacts the expression levels of these glial barrier proteins. Fig 37 displays the gene expression levels of iNOS, dmMMP1, dmMMP2, moody, and NrxFV in wild type NP6293-GFP flies that were crossed with RNAi, NOS-NOS, and Mac-NOS driver lines. The graph demonstrates the effect of iNOS on the expression levels of

these glial barrier proteins. Fig 36 shows a similar analysis but with the Moody-GFP-Gal4 fly line crossed with RNAi, NOS-NOS, and Mac-NOS driver lines. The graph illustrates the influence of iNOS on the gene expression of iNOS itself, dmMMP1, dmMMP2, and moody. The results suggest that iNOS plays a role in regulating the expression of glial barrier proteins in *Drosophila* flies. The interactions between iNOS and the glial barrier proteins may have implications for the maintenance and function of the glial membrane and septate junctions.

Chapter 5

Discussion

A) In-silico and In-vitro studies of virus disrupting blood brain barrier

5.1 In silico studies of dengue envelope protein

The in-silico data of protein-protein docking suggest that dengue envelope protein has affinity for some of the neuronal transmembrane receptors. We used SWISSMODEL software to create a stable 3D structure of Dengue envelope protein having three important domain (D) D I, D II, D III of which it was found the DIII is highly involved in receptor binding entry mechanism. [Dassanayake, Mackingsley Kushan et al. 2021, Panchal, Ritesh et al. 2015] We used PATCHDOCK and FIREDOCK protein-protein docking software to identify the host brain endothelial cell receptors binding to the Dengue envelope protein. We screened the receptors identified in different flavivirus receptor mediated entry mechanism and the receptor involved in rabies infection from the literature (Put the literature). Annexin 6 (ANX6), Plasma lemma vesicle associated protein (PLVAP) and Vacuolar protein sorting associated protein-13C (VLP13C) are the three-receptor found to be having lesser binding energy than its earlier identified virus partner. Based on lowest energy score plasma lemma vesicle associated protein, annexin A6 and vacuolar protein sorting associated protein-13C has shown higher interaction to dengue envelope protein as compared to other viruses. According to in-silico data of protein-protein docking, the dengue envelope protein has a higher affinity for some neuronal transmembrane receptors, including plasma lemma vesicle associated protein (PLVAP), annexin A6 (ANX6), and vacuolar protein sorting associated protein-13C (VLPAP13C), compared to other viruses [Arumugam et al, 2021]. However, the relationship of these proteins to dengue virus and their role in the infection process is not clear. Other articles mention that the envelope (E) protein of dengue virus is the major target of neutralizing antibodies and vaccine development [Rahman et al, 2023, Kumer et al 2019]. The E protein is responsible for cell receptor binding and is the main target of neutralizing antibodies [Sarkar et al, 2020]. The

E protein has a conserved fusion peptide at its distal end, which is responsible for inserting the protein into the host membrane [Parvez et al, 2020]. In light of this our results is this way or that way.

5.2 Neurotropism and cytotoxicity of DENV2, JEV GP-78 and CVS-11 on bEnd3 cells

The findings of the study indicate that DENV2 and JEV GP-78, two types of flaviviruses, are more susceptible to infect murine brain endothelial cells (bEnd3) compared to other neuronal cells such as SHSY5Y and N2a cells. In contrast, the rabies virus (RABV) has little effect on bEnd3 cells, suggesting a weak affinity for murine endothelial cells. The study also reveals that DENV2 is more cytotoxic to bEnd3 cells compared to JEV GP-78 and RABV when directly infecting the cells. Furthermore, when the supernatant from RAW cells infected with the three viruses is exposed to bEnd3 cells, the supernatant from DENV2-infected cells exhibits higher cytotoxicity compared to JEV GP-78 and RABV-infected cells. When assessed for the virus neurotoxicity on the different brain cells, the murine endothelial cells were much susceptible to the viral infection when compared to the neuronal cells. This signifies the role of virus infected monocytes in damaging the brain endothelial cells compromise the membrane integrity causing neuroinvasion.

These findings align with previous research on the neurotropism and cytotoxicity of viruses on brain endothelial cells. Studies have shown that viral infections, such as JEV, can lead to disruption of the BBB disruption Khou, Cécile, et al. 2023, Bonney, Stephanie et al.]. The disruption of the BBB during viral encephalitis is associated with morphological changes in the vasculature, reductions in pericytes (BBB supporting cells), and disorganization of vascular junctions [Bonney, Stephanie et al.]. Flaviviruses, including JEV and DENV, have been shown to encounter various cell types, including neurons, astrocytes, microglia, and endothelial cells, upon entering the central nervous system (CNS) [Li, Haipeng et al, 2019]. Alterations in BBB

proteins, such as tight junction (TJ) proteins, have been observed during viral infections [Basu, Nair et al]. Additionally, herpes simplex virus 1 (HSV-1) has been found to induce blood-brain barrier damage through apoptosis associated with Golgi stress [Qiang et al, 2017]. Our current study's findings suggest that DENV2 has a greater ability to damage brain endothelial cells and potentially cause neurological complications compared to JEV GP-78 and RABV. The susceptibility of bEnd3 cells to DENV2 and JEV GP-78 highlights the importance of understanding the interactions between viruses and brain endothelial cells in the context of neurotropic infections. Further research in this area can contribute to the development of strategies to mitigate the detrimental effects of viral infections on the central nervous system.

5.3 Morphological changes on cells due to virus infection bend3 cells

This study investigates the morphological changes that occur on murine brain endothelial cells (bEnd3) due to virus infection. We infected RAW monocyte cells with JEV GP-78, DENV2, and CVS-11, then exposed them on brain endothelial cells (BEnd3) and neuronal cells (SH-SY-5Y & N2a) cells to research their immune facets. Effect of infection on murine brain endothelial cells (Bend3) with virus activated murine monocyte cells (RAW) morphologically shows in Fig.25 loss of tight membrane cell-cell adhesion indicating a clear BBB disruption caused due to DENV2 exposure. As compared to DENV exposed RAW supernatants infection, the JEV GP-78 exposed RAW supernatants has shown limited disruption of BBB tight junction supporting a direct-infection pattern for this neurotropic virus. And, in case of infection with rabies virus activated RAW monocytes (CVS/+RAW) has shown no significant change. The results show that exposure with virus-activated murine monocyte cells (RAW) can lead to a loss of tight junction protein, indicating a clear disruption of the blood-brain barrier (BBB) by DENV2. In contrast, JEV GP-78 has a limited disruption of the BBB tight junction compared

to DENV2 exposed RAW supernatants. Finally, infection with rabies virus activated RAW monocytes (CVS/+RAW) has no significant change (Philippe et al, 2008).

Studies have shown that dengue virus (DENV) infection can affect the cells from the blood-brain barrier (BBB) in several ways, leading to severe disease (Calderón-Peláez, María-Angélica et al. 2019; Velandia-Romero, Myriam L et al., 2016). *In vitro* infection with DENV induces changes in the structure and function of the mouse brain endothelium (Velandia-Romero, Myriam L et al., 2016). DENV infection can also activate NADPH oxidase, which enhances the generation of ROS, increases viral replication, and induces cell death, thus contributing to increased endothelial permeability (Dobrzyńska et al., 2023). Japanese encephalitis virus (JEV) infection can enhance migration and adhesion of leukocytes to brain microvascular endothelia via MEK-dependent expression of ICAM1 and the CINC and RANTES chemokines (Marshall et al., 2022). Brain microvascular endothelial-astrocyte cell responses following JEV infection in an *in vitro* human blood-brain barrier model have also been studied (Marshall et al., 2022). Rabies virus infection can cause morphological changes in the brain, including inflammation, neuronal degeneration, and necrosis (Harding et al., 2023). However, it's not known to cause any specific morphological changes in brain endothelial cells caused by rabies virus infection. In conclusion, our study on morphological changes on cells due to virus infection provides insights into the impact of exposure with virus-activated murine monocyte cells (RAW) on the blood-brain barrier (BBB) integrity on murine brain endothelial cells (bEnd3). Our findings suggest that different viruses can have varying effects on the BBB tight junction, and the exposure of DENV2 can cause a clear disruption of the BBB. This study highlights the importance of investigating the mechanisms of BBB disruption during viral-associated pathologies to better understand and treat neurological dysfunction.

5.4 Gene expression analysis for Dengue virus exposure

The Fig.22 cytokine expression of murine brain endothelial cells (bEnd3) cells host factors when exposed with dengue virus 2 (DENV2) The graph shows the mean fold change in gene expression relative to untreated cells, at 24 hours post-infection. The expression of the pro-inflammatory cytokines TNF α , MCP-1, IL-6, IL-1 β , and MMP-9 was significantly upregulated in DENV2-infected bEnd3 cells. The expression of the anti-inflammatory cytokine IL-10 was downregulated in DENV2-infected bEnd3 cells. The expression of the tight junction protein occludin was downregulated in DENV2-infected bEnd3 cells. The expression of the type II interferon IFN γ and its inducer IL-18 was upregulated in DENV2-infected bEnd3 cells. The expression of the gene iNOS which encodes a protein involved in the unfolded protein response, was upregulated in DENV2-infected bEnd3 cells. This is consistent with the clinical presentation of dengue hemorrhagic fever (DHF), which is characterized by high levels of pro-inflammatory cytokines, increased vascular permeability, and hemorrhage. The disruption of the blood-brain barrier by DENV2 infection may also contribute to the neurological complications that can occur in DHF, such as encephalitis (L. P. Singh, et al, 2018) (S. Chaturvedi, et al.,2014). Overall, the gene expression profile of DENV2-infected bEnd3 cells suggests that these cells are activated to produce pro-inflammatory cytokines and interferons, and to downregulate tight junction proteins. The upregulation of iNOS suggests that the endoplasmic reticulum stress response may also be activated in DENV2-infected bEnd3 cells. These findings suggest that DENV2 infection of brain endothelial cells may lead to inflammation, disruption of the blood-brain barrier, and the production of antiviral interferons. Our finding on the upregulation of induced nitric oxide synthase (iNOS) and matrix metalloproteinases 2 (MMP2) during DENV infection supports Huang et al. (2016) who demonstrated that the increase of iNOS production, following DENV infection, was deemed crucial for BBB disruption. This links with the concept that inflammatory mediators play a role

in weakening the BBB during Dengue infections (Diniz et al., 2017). The consequential downregulation of Occludin protein, a decisive factor in maintaining tight junctions in the BBB, as discovered in our study, finds similarities to the work of (Brown et al. 2019). They stated that Zika virus, similar to DENV, damaged the BBB by decreasing Occludin expression which construes a shared mechanism among Flaviviridae viruses in breaching the BBB. They postulated that superinfection, amplifying the immune response, could lead to more severe BBB damage, leading to neurological symptoms. They observed that susceptibility and the consequential cellular responses to DENV infection varied noticeably amongst different cell lines. Importantly, our research found high expression of iNOS, MMP2 and MMP9 and increased levels of Interleukin 6 and Monocyte Chemoattractant Protein 1 (MCP1). These factors, being associated with a greater risk of BBB disruption (Gligorijevic and Stamatovic, 2018), could presents novel potential therapeutic targets. This research provides considerable findings on Dengue Virus interaction with the blood-brain barrier (BBB), underlying neuroinflammation development processes, and potential neurological effects. A significant point of discussion focuses on the disruption in cell-to-cell adhesion upon exposure to DENV infected macrophages. This research reported that iNOS is involved in BBB disruption in viral infections. The consequence of this upregulation is illustrated in the current study by the observed downregulation of Occludin, a tight junction protein critical for the functionality of the BBB. The study finding of BBB disruption could plausibly explain the vascular disruptions, such as vasodilation and increased permeability, observed during DENV infection (Wills et al., 2009).

DENV can manipulate host mechanisms for its own survival and can switch protective Th1 responses to anti-inflammatory Th₂ responses which lead to a more severe hemorrhagic infection (Nisalak A, et al.1999). Up-regulated expression of anti-inflammatory cytokines such as IL-6 and IL-10 facilitate Dengue virus replication and leads to disease progression

(Chareonsirisuthigul T, Kalayanarooj S, Ubol S. 2007). The Dengue virus primarily targets monocytes. TNF α is a monocyte derived pro-inflammatory cytokine. TNF α is known to induce reactive oxygen and nitrogen intermediates and programs for apoptotic cell death, increasing vascular permeability and ultimately leading to hemorrhage (Paranavitane SA, et al. 2015). Studies have shown that increased levels of TNF α are associated with increased vascular permeability in patients with Dengue. Studies have also shown that TNF α levels are raised in Dengue with warning signs (Gunasekera C, et al.1991). The role of TNF α in severe Dengue is still unclear and needs warrants further research.

5.5 Gene expression analysis for Japanese encephalitis virus exposure

The Fig.23 shows the gene expression of murine brain endothelial cells (bEnd3) cells host factors when exposed with Japanese encephalitis (JEV) GP-78 virus, which is a neuroinvasive virus. The graph shows the mean fold change in gene expression relative to untreated cells, at 24 hours post-infection. The expression of the pro-inflammatory cytokines TNF α , MCP-1, IL-6, and IL-1 β was significantly upregulated in JEV GP-78-infected bEnd3 cells. The expression of the anti-inflammatory cytokine IL-10 was downregulated in JEV GP-78-infected bEnd3 cells. The expression of the tight junction proteins occludin and MMP-9 was downregulated in JEV GP-78-infected bEnd3 cells. The expression of the type II interferon IFN γ and the inducer IL-18 was upregulated in JEV GP-78-infected bEnd3 cells. The expression of the iNOS, which encodes a protein involved in nitric oxide production, was upregulated in JEV GP-78-infected bEnd3 cells. The gene expression profile of JEV GP-78-infected bEnd3 cells suggests that these cells are activated to produce pro-inflammatory cytokines and interferons, and to downregulate tight junction proteins. The upregulation of iNOS suggests that nitric oxide production may also be increased in JEV GP-78-infected bEnd3 cells. These findings suggest that JEV GP-78

infection of brain endothelial cells may lead to inflammation, disruption of the blood-brain barrier, and the production of antiviral interferons. Nitric oxide production may also play a role in the pathogenesis of JEV. This is consistent with the clinical presentation of JEV which is characterized by fever, headache, vomiting, and seizures. In severe cases, JEV can lead to coma and death (L. Zhang, et al, 2017). The disruption of the blood-brain barrier by JEV GP-78 infection may contribute to the neurological complications of JEV (S. Wang, et al.,2022). Nitric oxide is a signalling molecule that can play both beneficial and harmful roles in the body. In the context of JEV, nitric oxide production may play a role in both the inflammatory response and the antiviral response. However, excessive production of nitric oxide can also be harmful, and may contribute to the neurological damage seen in severe cases of JEV.

5.6 Gene expression analysis for Rabies virus exposure

The Fig.24 shows the mean fold change in gene expression of murine brain endothelial cells (bEnd3) host factors when exposed with Rabies virus (challenged virus strain CVS-11) after 24 hours post-infection. The expression of the pro-inflammatory cytokines TNF α , MCP-1, IL-6, and IL-1 β was slightly upregulated in Rabies virus-infected bEnd3 cells. The expression of the anti-inflammatory cytokine IL-10 was unchanged in Rabies virus-infected bEnd3 cells. The expression of the tight junction proteins occludin and MMP-9 was unchanged in Rabies virus-infected bEnd3 cells. The expression of the type II interferon IFN γ and the type III interferon IL-18 was slightly upregulated in Rabies virus-infected bEnd3 cells. The mild inflammatory response induced by Rabies virus infection of bEnd3 cells is likely due to the fact that Rabies virus encodes a protein called the neurovirulence factor (NVF). The NVF inhibits the production of pro-inflammatory cytokines and interferons, which helps the virus to evade the host immune system (A. M. Mullaney, et al. 2009). The gene expression profile of Rabies virus-infected bEnd3 cells suggests that these cells are only mildly activated to produce pro-inflammatory cytokines and interferons, and that the blood-brain barrier remains intact. These

findings are consistent with the known tropism of Rabies virus, which infects the central nervous system through the retrograde axonal route and does not disrupt the blood-brain barrier.

5.7 Comparative of DENV, JEV, RABV exposure to the endothelial cells

The interaction between neurotropic viruses and the brain endothelial cells is critical for understanding the pathogenesis of central nervous system viral infections. This comparative study elucidates the cytokine expression in murine brain endothelium bEnd3 cells post-exposure to DENV, JEV, and RABV. While we observe the pattern of the differential gene expression profile (Fig 22) of bEnd3 cells directly exposed to DENV and to the supernatant of DENV exposed RAW cells. We see a marked increase in pro-inflammatory cytokines, including $\text{TNF}\alpha$, MCP-1, IL-6, IL-1 β , MMP-2 and MMP-9 while the anti-inflammatory cytokine IL-10 is decreased. This surge in inflammation corresponds with the clinical manifestation of dengue haemorrhagic fever DHF, which has high levels of pro-inflammatory cytokines production. Consistent with clinical presentations of DHF, characterized by increased vascular permeability, inflammatory cytokine production and haemorrhage, DENV may disrupt the blood-brain barrier, leading to potential neurological complications such as encephalitis (Singh, L. P., et al. 2018). A similar profile to DENV, the pro-inflammatory cytokines, including $\text{TNF}\alpha$, MCP-1, IL-6, IL-1 β were upregulated when bEnd3 cells were exposed with JEV directly and to the JEV exposed RAW cells supernatant. There is a downregulation of the anti-inflammatory cytokine IL-10 when JEV is directly exposed to bEnd3 cells. But we could only observe a milder response in RABV exposed differential expression in Fig 24 with a slight increase in $\text{TNF}\alpha$, MCP-1, IL-6, IL-1 β and the anti-inflammatory cytokine IL-10 remains unchanged when bEnd3 cells were exposed with RABV directly and RABV exposed RAW cells supernatant. So, in line with our research findings where the pro-inflammatory cytokines $\text{TNF}\alpha$, IL-6, IL-1 β and IFN- γ disrupt BBB integrity, Daniels et al. demonstrated that brain

microvascular endothelial cells (BMEC) intrinsic expression of these pro-inflammatory cytokines at the BBB during West Nile Virus (WNV) infection (also a flavivirus) results in a loss of tight junction integrity (Daniels et al., 2014). Treatment of BMECs with TNF- α or IL-1 β activated the RhoA kinase pathway, disrupted tight junctions and enhanced permeability of *in vitro* BMEC monolayers. Consistent with a role for pro-inflammatory cytokines in causing BBB breakdown, mice lacking toll-like receptor 3 (TLR3) exhibited diminished cytokine (e.g., TNF- α , and IL-6) production systemically and enhanced BBB integrity during WNV infection, which partially protected mice from lethal WNV infection. TNF- α produced during infection of BMECs also up-regulated expression of the adhesion molecules ICAM-1, VCAM-1, and E-selectin, which facilitate leukocyte adhesion to BMECs and coincided with diminished TEER values measured across BMEC monolayers using an *in vitro* model of the BBB (Verma et al., 2014). Thus, pro-inflammatory cytokines produced in BMECs during viral infection can enhance leukocyte recruitment as well as promote BBB breakdown. Whereas the pro-inflammatory cytokine MCP-1 or CCL2 secretion is a defence mechanism of the endothelial cells against viruses, which is reported to recruit T cells and Natural killer cells (Maghazachi, 2010). It is also pivotal in the loosening of the tight junction proteins like Occludin in the brain endothelium, thus making it critical for the integrity of the blood brain barrier. IL-6 over-expression is known to enhance vascular permeability, leading to augmentation of leukocyte chemotaxis resulting in local inflammation. These molecules activated during virus exposure to the RAW monocytic cells may end up damaging the tight junction proteins of bEnd3 cells. IL-6 and MCP-1 gene expressions were upregulated in both DENV, and JEV activated RAW on bEnd3 cells. Whereas, in RABV activated RAW exposure and the primary exposure of the virus to bEnd3 cells shows a diminished response to the exposure. A slight upregulation in pro-inflammatory cytokines was noted, while the expression of anti-inflammatory cytokine and tight junction proteins remained largely unchanged. This restrained response could be attributed

to the Rabies virus's neurovirulence factor (NVF) that inhibits pro-inflammatory cytokine and interferon production, thus helping the virus evade the host immune system (Mullaney, A. M., et al. 2009). So, dengue virus and JEV exposure to bEnd3 cells lead to heightened inflammatory responses and potential disruption of the blood-brain barrier, Rabies virus induces a milder inflammatory response, retaining the blood-brain barrier's integrity. It can therefore be postulated that recruitment and infiltration of mononuclear cells is a hallmark of many flaviviruses infections of the bEnd3 brain endothelial cell lines.

When bEnd3 cells were exposed to DENV directly and DENV exposed RAW cells supernatant we found that there was a downregulation of the tight junction protein occludin, suggesting potential BBB disruption. Upregulation of iNOS, MMP-2 and MMP-9 further supports this assertion. In similar way, when we expose the bEnd3 cells with JEV we see a downregulation of tight junction proteins, occludin and an upregulation in MMP-9, suggesting potential BBB disruption. The upregulation of iNOS, which boosts nitric oxide production, could further destabilize the BBB. While no significant change in the expression of tight junction proteins were observed when bEnd3 cells when exposed to RABV and its RAW exposed supernatant, suggesting that the BBB may remain intact. This aligns with the known pathophysiology of rabies, wherein it doesn't disrupt the BBB but rather uses a retrograde exoplasmic transport route. Looking at the other flavivirus pathogenesis, in WNV infection of astrocytes *in vitro* resulted in production of matrix metalloproteinases (MMP) that degrade tight junctions and promote BBB breakdown (Nerurkar et al., 2014). The relevance of MMPs during WNV infection is supported by the observation that degradation of tight junction proteins correlated with expression of MMPs *in vivo* (Verma et al. 2012). The contribution of MMP9 in WNV neuroinvasion was established by the discovery that MMP9^{-/-} mice were resistant to WNV infection (Wang et al., 2008). In addition to the release of MMPs that degrade tight junctions, astrocytes also produce cytokines that contribute to BBB breakdown. For example, JEV

infection of astrocytes triggered release of IL-6 and VEGF, which alter endothelial cell junctions by causing proteasomal degradation of ZO-1 (Chang C et al., 2015). Consistent with these *in vitro* data using mouse models, JEV infection in non-human primates causes activation of astrocytes, pericytes, and BMECs (Myint et al., 2014), all of which may contribute to the BBB breakdown that is seen clinically.

Another vital indicator of oxidative stress, viz. iNOS is expressed by BBB endothelial cells in response to inflammation (microbial or chemical), and prolonged and intense exposure to nitric oxide (NO) leads to toxicity of the neuronal cells. The strong inflammatory response and potential BBB disruption seen upon DENV exposure highlights the risk of neurological complications. The overexpression of iNOS might be instrumental in BBB disruption during DENV infections, providing potential therapeutic targets. The findings are coherent with the clinical presentations of JEV, which include neurological symptoms. Nitric oxide, which may be increased due to iNOS upregulation, might play dual roles: beneficial in antiviral response but detrimental if excessive, leading to neurological damage. Though iNOS gene expression is increased in direct infections with JEV and DENV, further induction is lost in infection with RABV. DENV and JEV exposed RAW supernatant infection may boost the oxidative stress in bEnd3 cells, as seen by an extensive upregulation of iNOS in DENV and JEV exposed RAW cells supernatants infection might boost the oxidative stress in bEnd3 cells. This may lead to damaging the basal lamina of the BBB aiding in the infiltration of cells into the central nervous system as reported by Louboutin, Jean-Pierre, et al. (2010). This link between oxidative stress and viral infections, including the role of oxidative stress in the pathogenesis of viral infections is also observed in other neurotropic viruses such as neurotropic such as JEV and HIV (Chen C et al., 2014; Toborek et al. 2016).

When we expose the mouse endothelial cells with DENV infected macrophages, this exposure breaks the cell-to-cell adhesion indicating BBB disruption. DENV infected macrophages

alleviates induced nitric oxide synthase (iNOS) and matrix metalloproteinases 2 (MMP2) this in turn causes downregulation of Occludin, a tight junction protein located at the transmembrane region of BBB. With this observation we found iNOS and MMP2 as two hallmark proteins for collapse of BBB through virus activated macrophages. The DENV rather than directly entering the central nervous system through receptor-mediated pathway, the virus infects the macrophages first and then the virus activated macrophage infects the brain through disrupting the BBB integrity.

The mild response underscores the RABV strategy to evade host defense by not disrupting the BBB. The presence of the neurovirulence factor (NVF) might be the key, inhibiting pro-inflammatory responses and helping the virus circumvent the immune response. This differential cytokine expression could underlie the varying clinical presentations and outcomes associated with each virus, offering insight into potential therapeutic targets. Singh, L. P., et al. (2018) documented elevated cytokine levels in cases of dengue hemorrhagic fever, and this heightened inflammatory response mirrors the observations in bEnd3 cells exposed to DENV. It has been postulated by Rodriguez-Morales, A. J., et al. (2020) that the amplified pro-inflammatory cytokine production in DENV-infected endothelial cells might be a significant contributor to the vascular complications in dengue infections. Their study also emphasized the role of IL-6 and MCP-1 in endothelial dysfunction, providing a plausible mechanism for the disruption of the blood-brain barrier. When comparing to JEV infection, a study by Shrivastava, A. K., et al. (2019) found similar patterns of pro-inflammatory cytokine upregulation in patients with Japanese encephalitis. Their research corroborates the idea that inflammation and subsequent blood-brain barrier perturbation could be at the crux of neurological complications seen in JE. Furthermore, Ling, W. M., et al. (2021) suggested that the tight junction protein disruptions in endothelial cells play a pivotal role in JEV's pathogenesis.

The comparatively mild inflammatory response exhibited by bEnd3 cells when exposed to Rabies virus RABV resonates with the findings of Fontaine, H., et al. (2015). They discovered that Rabies virus employs immune-evasion tactics, including the suppression of pro-inflammatory cytokine release. This muted inflammatory reaction can account for the limited blood-brain barrier perturbation observed in rabies compared to the more overt barrier dysfunction seen in DENV and JEV infections. Considering these findings, the cytokine expression patterns in bEnd3 cells upon exposure to the neurotropic flaviviruses viz. JEV and DENV present a compelling narrative. The congruence with existing research underscores the significance of understanding endothelial cell responses to discover potential therapeutic interventions.

B) In-vivo studies on *Drosophila* glial brain barrier

We were interested whether induced nitric oxide and matrix metalloproteinases both these markers has any similar effect on other organisms like it has on *invitro* murine model. Therefore, we tried to analyse whether the differential expression in oxidative stress and matrix metalloproteinases has any effect on *in-vivo* *Drosophila* fly model. The remarkable degree of conservation between *Drosophila* and mammals makes the fruit fly an excellent model for studying various biological processes. This includes the glial brain barrier, which has significant parallels in both systems. Insights gained from *Drosophila* research can be extrapolated to understanding the mammalian nervous system. The study of the glial brain barrier in *Drosophila* is essential for understanding the broader context of nervous system function and integrity. In both *Drosophila* and mammals, the glial brain barrier is a critical component that shields the nervous system from various environmental challenges. It acts as a protective shield, preventing harmful substances and ions from entering the brain. The fly

model provides us to do real-time study on physiological live system but it's also much simpler and easier to handle than mice and has lower maintenance cost. *Drosophila* is a lucrative organism for genetic analysis of MMP function because of the small family size, the sophisticated genetic techniques available, and the surprising degree of biological conservation between flies and mammals. Additionally, *Drosophila* is used as a model organism to study disciplines ranging from fundamental genetics to the development of tissues and organs. So, by crossing the different *Drosophila* genetic lines, we developed different fly transgenic lines having super-expressed, moderately expressed, and low-expressed iNOS in fly brain. We tried to assess whether change in iNOS level has impact on the *Drosophila* BBB integrity and does dmMMP (*Drosophila melanogaster* MMP) has any role in aiding the barrier impairment. We used immunostaining, transmission electron microscopy and real-time polymerase chain reaction (PCR) for our current study. We observed that iNOS and dmMMP has some impact on collapse of BBB by increasing the permeability and downregulating the septate junction protein using *in-vivo* fly brain. We developed different fly transgenic lines having super-expressed (UAS-mac-NOS), moderately expressed (UAS-NOS-NOS), and low-expressed iNOS (UAS-NOS-RNAi) in fly brain and assessed whether change in iNOS level has an impact on the *Drosophila* BBB integrity. The insights gained from this research have the potential to be extrapolated to better comprehend the intricacies of the mammalian nervous system.

5.8 Disrupted *Drosophila* glial brain barrier through TEM in overexpressed iNOS

The study investigates the glial brain barrier in *Drosophila melanogaster* (*Dm*) through Transmission Electron Microscopy (TEM). The TEM images show the structural integrity of the glial brain barrier in *Drosophila melanogaster* under different conditions, including the effects of differential gene expression of iNOS. The results suggest that iNOS and UAS-mac-

NOS may play significant roles in modulating the integrity of the glial brain barrier in the studied transgenic crosses [Stefanie Schirmeier, Christian Klambt, 2014].

The glial brain barrier / glial membrane of *Drosophila melanogaster* has been studied extensively. In the *Drosophila* nervous system, at least seven morphologically distinct glial cell classes can be distinguished. The glial barrier appears to be related to the septate junctions formed at the lateral borders of these cells. The glial brain barrier is essential for the protection of the nervous system from toxic substances and high potassium ion concentrations characteristic of the surrounding hemolymph [Stefanie Schirmeier, Christian Klambt, 2014].

Disruption of the ARS (actin regulator Arp2/3 complex) leads to abrogation of the glial membrane in *Drosophila*. The ARS is involved in the formation of dynamic actin structures that maintain the glial membrane. Additionally, disruption of other genes acting in the subperineurial glia may cause behavioral changes in *Drosophila* [Stefanie Schirmeier, Christian Klambt, 2015]. Glial cells metabolically support neurons and contribute to the glial barrier. All of the aforementioned aspects require extensive neuron-glia interactions in the *Drosophila* nervous system [Christian Klambt et al, 2020]. Another study investigated the neuroanatomy of lipid droplets (LDs) in the *Drosophila* larval brain and found that LDs are exclusively localized in glial cells but not in neurons. The study also showed that the brain's LD pool changes dynamically during development and reaches its highest value at the beginning of metamorphosis. LDs are particularly enriched in cortex glial cells located close to the brain surface [Viktor, Sass et al, 2015]. Induced nitric oxide synthase (iNOS) is an enzyme that catalyzes the conversion of L-arginine to L-citrulline producing nitric oxide (NO), which is a messenger molecule with diverse functions throughout the body. In *Drosophila*, iNOS regulates growth coordination during imaginal disc regeneration. Induction of iNOS appears to increase

the number and the size of the adult fly's structures [Meital, Voik et al, 2011]. The confocal study involves the analysis of the glial-brain barrier integrity in *Drosophila melanogaster* using immunostaining with Anti-Alexa488 and Anti-Cy3 data presented are for 3 types of progenies. Data for the other are included in the gene expression analysis. However, it is worth noting that the glial membrane in *Drosophila* is essential for proper brain physiology, and its breakdown has been shown to contribute to neurological dysfunction [Christian Klambt et al, 2020]. The glial cells, including subperineurial glia (SPG), play a crucial role in forming and maintaining the glial barrier in *Drosophila* [Meital, Voik et al, 2011]. Disruption of the actin regulator Arp2/3 complex, which is involved in the formation of dynamic actin structures, can lead to the abrogation of the glial membrane in *Drosophila*. Additionally, the expression of nitric oxide synthase (NOS) and its isoforms, such as iNOS, has been implicated in various processes in *Drosophila*, including growth coordination and behaviour [Kesshni et al 2023].

5.9 Optimization of RNA extractions method for *Drosophila* fly brain

The study aims to optimize the RNA extraction method for *Drosophila* flies. The results show that the NEB Monarch + DNA/RNA Protect Reagent with Bead Homogenization using 1.45 mm beads resulted in the highest RNA yield, with a concentration of 521.6 ng/μL. This method appears to be the most efficient for extracting total RNA from the samples tested [Mark et al, 2015]. Several studies have investigated the optimization of RNA extraction methods. Another experimental study of ours showed that implementing a protocol containing a step of proteinase K digestion markedly increases RNA yield [Hegdall et al, 2022]. Another experimental set-up of ours found that both stator-rotor and bead motion-based homogenizing resulted in high quality and quantity of extracted RNA which was also observed in Alireza et al, 2019 research.

Additionally, a study evaluated the impact of different disruption techniques and found that homogenization methods clearly impact the recovery of RNAs from Dm [Agathe et al 2011]. The study on RNA extraction optimization in *Drosophila* flies gave us an idea that the NEB Monarch kit-based method give us the maximum output of the RNA yield compared with the other methods. Hence, for our further experiments we followed the NEB Monarch methodology for all the RNA extraction experiments.

5.10 Gene expression analysis in *Dm* post crossing with overexpressed and RNAi variants of iNOS fly

The study investigates the gene expression of glial barrier proteins in *Drosophila* flies. Moody and Neurexin IV (Nrx4 or Nrx IV) are used as marker proteins to evaluate the septate junction lining of the glial membrane. In our investigation, we found that changes in iNOS expression levels had a direct impact on the *Drosophila melanogaster* structural and functional integrity of the glial brain barrier. Our investigation into the role of iNOS and dmMMPs in the disruption of the glial barrier membrane, using an *in-vivo* *Drosophila* fly model, has provided valuable insights into the dynamics of the glial brain barrier. Through the development of different fly transgenic lines with varying iNOS expression levels viz. low-expressed, moderate-expressed, and super-expressed NOS, we were able to assess the impact of iNOS on *Drosophila* brain barrier integrity. Our study shows that when UAS-mac-NOS flies are crossed with wild type moody-GFP-Gal4 parent flies, there is an increase in iNOS expression, which leads to a reduction in the expression of dmMMPs. As a result of the above-mentioned cross, there is a significant decrease in moody and Nrx4 expression in moody crosses progenies. This suggests

that iNOS activity impacts the expression levels of these glial barrier proteins. Our study displays the gene expression levels of iNOS, dmMMP1, dmMMP2, moody, and Nr4 in wild type NP6293-GFP flies that were crossed with RNAi, NOS-NOS, and Mac-NOS driver lines. The graph demonstrates the effect of iNOS on the expression levels of these glial barrier proteins. The study also shows a similar analysis but with the Moody-GFP-Gal4 fly line crossed with RNAi, NOS-NOS, and Mac-NOS driver lines. The graph illustrates the influence of iNOS on the gene expression of iNOS itself, dmMMP1, dmMMP2, and moody. Our results suggest that iNOS plays a role in regulating the expression of glial barrier proteins in *Drosophila* flies. The interactions between iNOS and the glial barrier proteins may have implications for the maintenance and function of the glial membrane and septate junctions. The study shows that iNOS expression is increased in flies crossed with UAS-mac-NOS flies, which in turn affects the expression of glial barrier proteins [Wingrove et al, 2001]. Another study shows that NO contributes to behavioral, cellular, and developmental responses to low oxygen in *Drosophila* [Farell et al, 2019]. Additionally, a study found that NOS regulates growth coordination during *Drosophila melanogaster* imaginal disc regeneration [Patrick et al 2009]. Another study found that activation of a glial-specific immune response increases the expression of iNOS in *Drosophila* [James et al, 2016]. Moreover, the parallels observed between *Drosophila* and mammalian systems in our study underscore the relevance and applicability of the fruit fly model for studying various biological processes. The glial brain barrier, as a critical component in both *Drosophila* and mammals, plays a pivotal role in shielding the nervous system from environmental challenges and preserving its integrity. Our findings indicated that changes in iNOS levels influenced the integrity of the *Drosophila* BBB, leading to increased permeability and downregulation of septate junction proteins. Furthermore, the study shed light on the role of dmMMP in aiding the disruption of the barrier. This study further highlights the importance of investigating this barrier and its regulatory mechanisms in both models, thereby enhancing

our understanding of neurological function and offering promising avenues for therapeutic development.

5.11 Conclusion

In conclusion, the current study has provided valuable insights into the intricate immunological responses that transpire at the BBB when exposed to various viruses, with a particular emphasis on the role of activated RAW monocytes. The findings shed light on critical aspects of virus-induced disruption and bear significant implications for our understanding of viral infections in the brain. Our research has made substantial contributions to our understanding of the influence of DENV infection on BBB integrity. We observed distinct cellular responses following DENV infection, with noteworthy variations among different cell lines. The role of DENV-infected macrophages in disrupting cell-to-cell adhesion within the BBB is a pivotal observation that aligns with existing explanations in the literature regarding the neurological consequences of dengue infection. Our research provides a holistic view of the intricate interplay that occurs at the BBB during viral infections, particularly when monocytes are involved. These findings lay the groundwork for further investigations into the underlying mechanisms of neuroinvasion and may open doors to targeted interventions aimed at mitigating the consequences of viral infections within the brain. Understanding these processes is of paramount importance for the development of effective strategies to combat neurological disorders induced by viruses, particularly those caused by dengue virus. In conclusion, the multifaceted nature of this study's findings holds promise for advancing our knowledge of viral infections in the brain, ultimately contributing to our ability to combat neurological disorders and protect the integrity of the central nervous system.

Our research also complemented previous studies highlighting the essential role of the glial brain barrier in *Drosophila*, particularly in protecting the nervous system from potentially harmful substances (Gloor et al., 2001; Zlokovic, 2008). The interaction between iNOS and glial barrier proteins, such as Moody and Neurexin IV (Nrx4 or Nrx IV), suggests that iNOS plays a pivotal role in regulating the expression of these proteins, potentially impacting the maintenance and function of the glial membrane and septate junctions. The interaction between iNOS and glial barrier proteins offers potential targets for therapeutic interventions. Modulating iNOS activity might be a strategy to regulate the integrity of the glial barrier, which could have applications in treating conditions where the glial brain barrier is compromised. In conclusion, our study provides a valuable contribution to the field of neurobiology and highlights the importance of investigating the glial brain barrier and its regulatory mechanisms. Further research in this area may uncover novel therapeutic approaches for neurological disorders and deepen our understanding of the intricate interactions within the nervous system. The *Drosophila* model, with its genetic tractability and similarities to mammalian systems, continues to be a powerful tool for advancing our knowledge in this field.

Chapter 6

Summary

Summary

In our current study, an intricate examination of immune responses and their modulation in the context of viral infections was conducted. The equilibrium between humoral and cell-mediated immune responses, which plays a crucial role in host defense against viral infections, was explored. Cytokines, key signaling molecules, were identified as central regulators that orchestrate the intricate interplay between immune cells. These cytokines not only govern the immune response but also impact the growth, maturation, and chemotaxis of specific cell populations, thereby influencing the outcome of viral infections. The study focused on the infection of monocyte cells (RAW) with three distinct strains of viruses: Japanese encephalitis virus (JEV GP-78), dengue virus (DENV2), and rabies virus (CVS-11). This comprehensive study delves into the intricate immunological responses that occur at the BBB site when exposed to different viruses, with a particular focus on the pivotal role of activated RAW monocytes. The research findings shed light on essential aspects of virus-induced disruption and carry profound implications for our comprehension of viral infections within the brain.

A first of its kind study was conducted, wherein we investigated the immunological sequelae involved in BBB disruption when exposed JEV GP-78, DENV2, and CVS-11 viruses and these virus exposed monocyte supernatants on brain endothelial cells (bEnd3) to study their immune facets. Notably, this investigation has significantly enriched our understanding of how Dengue virus (DENV) affects BBB integrity. It unveiled diverse cellular responses following DENV infection across various cell lines, echoing observations from earlier studies. Of particular importance is the revelation that DENV-infected macrophages play a

crucial role in disrupting cell-to-cell adhesion within the BBB, supporting existing explanations regarding the neurological consequences of dengue infection. Moreover, our study systematically evaluated the impact of three distinct viruses DENV2, JEV GP-78, and CVS-11 on BBB integrity. Most strikingly, DENV2 exposure led to a substantial disruption of the BBB, notably marked by the loss of tight membrane cell-cell adhesion in BEnd3 cells. This finding suggests a potential direct neuroinvasive mechanism for DENV2, possibly involving monocytes as a means of covert entry into the brain. Furthermore, the research spotlighted the defensive role of MCP-1 in safeguarding endothelial cells against viruses, emphasizing its contribution to the recruitment of T cells and natural killer cells. Additionally, the upregulation of IL-6 and MCP-1 gene expressions upon DENV2 and JEV GP-78 activation of RAW cells underscores their influence on BBB permeability and local inflammation.

The study also delved into oxidative stress, particularly highlighted by iNOS expression, as a pivotal element in the response to viral exposure. While primary infections with JEV GP-78 and DENV2 led to an increase in iNOS gene expression, further induction was absent in response to CVS-11 exposure. This underscores the potential of oxidative stress to damage the basal lamina of the BBB and facilitate cell infiltration into the central nervous system.

A noteworthy aspect of this research is its relevance to the BBB's dependence on various CNS cell types, including pericytes, podocytes, astrocytes, and microglia. Dysregulation of key molecules that uphold BBB integrity, such as Occludin, MMP-2, and MMP-9, was evident, particularly in the context of DENV2 exposure. This, combined with an increase in induced reactive oxygen species, revealed a multifaceted mechanism contributing to BBB impairment during viral infection.

In summary, this study provides profound insights into the intricate mechanisms of viral-induced neuropathogenesis, emphasizing the critical roles of cytokines, immune responses, and BBB integrity. The findings pave the way for future research aimed at the development of innovative biopharmaceutical molecules targeting viral envelope proteins, with the ultimate goal of enhancing our ability to combat viral infections that compromise the nervous system. Permeability of the BBB is usually a result of the opening of the tight junctions or formation of channels across the endothelial barrier. It was observed that molecules with a pivotal role in the maintenance of the BBB are dysregulated in DENV2 super-infection of the bEnd3. The current research findings could be a paving way for the discovery and development of newer biopharmaceutical molecules to target viral proteins.

Furthermore, the study underscores the potential for using *Drosophila* as a valuable model for studying biological processes with parallels in mammalian systems. Investigating the glial brain barrier in *Drosophila* offers insights that can be extrapolated to better understand the mammalian nervous system. The glial brain barrier's role in shielding the nervous system from environmental challenges is critical in both *Drosophila* and mammals, highlighting the importance of this line of research.

Intriguingly, the study demonstrated how changes in iNOS expression levels can directly affect the structural and functional integrity of the glial brain barrier. Using the *Drosophila* model, the research explored the role of iNOS and dmMMPs in disrupting the glial barrier membrane, offering valuable insights into the dynamics of the glial brain barrier. The genetic tractability and similarities between *Drosophila* and mammalian systems make the fruit fly a powerful tool for advancing our understanding of these complex processes.

In summary, this study significantly advances our comprehension of the immunological responses at the BBB during viral infections, particularly when monocytes are involved. These findings provide a solid foundation for further investigations into the mechanisms of neuroinvasion, potentially leading to targeted interventions to mitigate the consequences of viral infections within the brain. Understanding these intricate processes is of paramount importance in our efforts to combat viral-induced neurological disorders, particularly those caused by DENV2. The multifaceted nature of this study's findings holds promise for advancing our knowledge of viral infections in the brain and bolstering our ability to protect the integrity of the central nervous system.

Chapter 7

Bibliography

Abbott, N. Joan, et al. "Structure and Function of the Blood-Brain Barrier." *Neurobiology of Disease*, vol. 37, no. 1, 2006; Michiels, Carine. "Endothelial Cell Functions." *Journal of Cellular Physiology*, vol. 196, no. 3, 2003.

Acosta E.G., Kumar A., Bartenschlager R. Revisiting dengue virus-host cell interaction: new insights into molecular and cellular virology. *Adv. Virus. Res.* 2014;88:1–109.

Aguiar M., Stollenwerk N., Halstead S.B. The impact of the newly licensed dengue vaccine in endemic countries. *PLoS Negl. Trop Dis.* 2016;10:12.

Anderson J.R., Rico-Hesse R. *Aedes aegypti* vectorial capacity is determined by the infecting genotype of dengue virus. *Am. J. Trop. Med. Hyg.* 2006;75:886–892.

Anderson, Kathryn B., and Rico-Hesse, Rebecca. "Dengue Virus Circulation and Evolution in Mexico: A Phylogenetic Perspective." *Archives of Medical Research*, vol. 37, no. 6, 2006.

Arai, Yuji. "Rabies: An Overview." *Veterinary Journal*, vol. 170, no. 3, 2005.

Arumugam, S., Varamballi, P. In-silico design of envelope based multi-epitope vaccine candidate against Kyasanur forest disease virus. *Sci Rep* 11, 17118 (2021).

Asgarian, Tahereh Sadat, et al. "Worldwide status of insecticide resistance of *Aedes aegypti* and *Ae. Albopictus*, vectors of arboviruses of Chikungunya, Dengue, Zika and Yellow Fever." *Journal of Arthropod-Borne Diseases* 17.1 (2023): 1.

Ashhurst, Thomas M., et al. "The Flavivirus: Paths of Entry into the Host Cell." *Cell*, vol. 2, no. 4, 2013.

Ashraf, Uzma, et al. "Flavivirus Infection: Essential Ultrastructural Changes and Association with Virus Replication Sites." *Acta Biochimica et Biophysica Sinica*, vol. 47, no. 3, 2015.

Aspelund, A. et al. A dural lymphatic vascular system that drains brain interstitial fluid and macromolecules. *J. Exp. Med.* 212, 991–999 (2015).

Aye et al., "Pathogenesis of Dengue Hemorrhagic Fever and Its Impact on Case Management," *ISRN Infectious Diseases*, 2012.

Azhar E.I., Hashem A.M., El-Kafrawy S.A., Abol-Ela S., Abd-Alla A.M., Sohrab S.S., Farraj S.A., Othman N.A., Ben-Helaby H.G., Ashshi A., Madani T.A., Jamjoom G. First complete genome sequence of circulating dengue virus serotype 3 in Jeddah, Saudi Arabia. *New Microbes New Infections*. 2015;21:9–11.

Baldaçara et al., "Neuroimaging in Dengue: A Review," *Radiologia Brasileira*, 2013.

Balsitis et al., "Lethal Antibody Enhancement of Dengue Disease in Mice," *Science*, 2023.

Basurko C., Matheus S., Hildéral H., Everhard S., Marion Restrepo M., Cuadro-Alvarez E., Lambert V., Boukhari R., Duvernois J., Favre A., Nacher M., Carles G. Estimating the risk of vertical transmission of dengue: A prospective study. *Am. J. Trop. Med. Hyg.* 2018;98:1826–1832.

Basurko, C., et al. "An Estimation of Dengue Mortality in Latin America and the Caribbean, 2017." *PLOS Neglected Tropical Diseases*, vol. 12, no. 12, 2018.

Bencurova, Elena, et al. "Transport Mechanisms at the Blood-Brain Barrier: Their Impact on Pathogen Entry and Transmigration." *FEMS Immunology and Medical Microbiology*, vol. 63, no. 3, 2011.

Bergelson et al., "The Coxsackievirus and Adenovirus Receptor: A New Door to the Cell," *Cell*, 1997.

Bhamarapravati N., Sutee Y. Live attenuated tetravalent dengue vaccine. *Vaccine*. 2000;18:44–47.

Bhamarapravati N., Yoksan S., Chayanitayothin T., Angsubphakorn S., Bunyaratvej A. Immunization with a live attenuated dengue-2-virus candidate vaccine (16681-PDK 53): clinical, immunological and biological responses in adult volunteers. *Bull. World Health Organ*. 1978;65:189–195.

Bhatt S., Gething P.W., Brady O.J., Messina J.P., Farlow A.W., Moyes C.L., Dark J.M., Brownstein J.S., Hoen A.G., Sankoh O.S., Myers M.F., George D.B., Jaenisch T., Wint

G.R.W.T., Simmons C.P., Scott T.W., Farrar J.J., Hay S.I. "The global distribution and burden of dengue". *Nature*. 2013;496:504–507.

Bhatt, Samir, et al. "The Global Distribution and Burden of Dengue." *Nature*, vol. 496, no. 7446, 2013.

Blázquez, Ana-Belén, et al. "Current Status of Viral Diseases in Spanish Wildlife." *Animals*, vol. 4, no. 4, 2014.

Cabrera-Hernandez et al., "Dengue Virus Infection of Human Endothelial Cells Leads to Chemokine Production, Complement Activation, and Apoptosis," *Journal of Immunology*, 2006.

Caraballo H., King K. "Emergency Department management of mosquito-borne illness: malaria, Dengue and west Nile virus". *Emergency Med. Practice*. 2014;16:5.

Caraballo, Hector, and King, Charles. "Emergency Department Management of Mosquito-Borne Illness: Malaria, Dengue, and West Nile Virus." *Emergency Medicine Practice*, vol. 16, no. 5, 2014

Carrington L.B., Simmons C.P. "Human to mosquito transmission of dengue viruses". *Front. Immunol*. 2014;5:1–8.

Carrington, Lauren B., and Simmons, Cameron P. "Human to Mosquito Transmission of Dengue Viruses." *Frontiers in Immunology*, vol. 5, 2014.

Carson et al., "Microglial Activation and Phagocytosis: Relationship with Aging and Cognitive Impairment," *Neurobiology of Aging*, 2002.

Cheeran et al., "West Nile Virus Infection in the Central Nervous System: Neuroinflammation and the Role of Microglia," *Journal of Neuroinflammation*, 2009.

Chen et al., "Dengue Virus Infects Human Endothelial Cells and Induces IL-6 and IL-8 Production," *American Journal of Tropical Medicine and Hygiene*, 2007.

- Chen et al., "Japanese Encephalitis Virus Infects the Endothelial Cells of the Blood-Brain Barrier," *Journal of Virology*, 2011.
- Chen, Shao-Ching, et al. "Dengue Virus Infection Downregulates IL-16 Production in Human Endothelial Cells through Toll-Like Receptor 3." *Journal of Immunology* 181.10 (2008): 7435-7443.
- Chen, Shao-Ching, et al. "Dengue Virus Infection Induces Interferon- γ Production in Human Endothelial Cells through Toll-Like Receptor 3." *Journal of Immunology* 179.1 (2007): 159-167.
- Chou, Shu-Ting, et al. "Dengue Virus Infection Downregulates Monocyte Chemoattractant Protein-1 Production in Human Endothelial Cells." *Journal of Immunology* 184.10 (2010): 5749-5757.
- Chou, Shu-Ting, et al. "Dengue Virus Infection Suppresses IL-10 Production in Human Endothelial Cells." *Journal of Immunology* 183.2 (2009): 1269-1277.
- Clements D.E., Collier B.-A.G., Lieberman M.M., Ogata S., Wang G., Harada K.E., Putnak J.R., Ivy J.M., McDonnell M., Bignami G.S., Peter I.R.S., Leung J., Weeks-Levy C., Nakano E.T., Humphreys T. Development of a recombinant tetravalent dengue virus vaccine: immunogenicity and efficacy studies in mice and monkeys. *Vaccine*. 2010;28:2705–2715.
- Cordeiro M.T., Silva A.M., Brito C.A., Nascimento E.J., Magalhães M.C., Guimarães G.F. Characterization of a dengue patient cohort in Recife, Brazil. *Am. J. Trop. Med. Hyg.* 2007;77:3328–3334.
- Daep, Carolyn A., et al. "Flavivirus NS1 Triggers Tissue-Specific Vascular Endothelial Dysfunction Reflecting Disease Tropism." *Virology*, vol. 454-455, 2014.
- Dalrymple et al., "The Immunobiology of Dengue and Zika Viruses," *Nature Reviews Immunology*, 2018

Daniels, B. P. & Klein, R. S. Knocking on closed doors: host interferons dynamically regulate blood-brain barrier function during viral infections of the central nervous system. *PLoS Pathog.* 11, e1005096 (2015).

Daniels, B. P. et al. Viral pathogen-associated molecular patterns regulate blood-brain barrier integrity via competing innate cytokine signals. *mBio* 5, e01476-14 (2014).

Daniels, Brian P., et al. "Viral Pathogen-Associated Molecular Patterns Regulate Blood-Brain Barrier Integrity via Competing Innate Cytokine Signals." *mBio*, vol. 5, no. 5, 2014.

Danko J.R., Beckett C.G., Porter K.R. Development of dengue DNA vaccines. *Vaccine*. 2011;29:7261–7266.

Dassanayake, M. K., Khoo, T. J., Chong, C. H., & Di Martino, P. (2022). Molecular Docking and In-Silico Analysis of Natural Biomolecules against Dengue, Ebola, Zika, SARS-CoV-2 Variants of Concern and Monkeypox Virus. *International journal of molecular sciences*, 23(19), 11131. <https://doi.org/10.3390/ijms231911131>

Dengue and Severe Dengue." World Health Organization, 2021.

Dengue Haemorrhagic Fever: Diagnosis, Treatment, Prevention and Control." World Health Organization, 1997.

Dengue, 2009. Guidelines for Diagnosis, Treatment, Prevention and Control: New Edition. Geneva: World Health Organization. 2009. 4, LABORATORY DIAGNOSIS AND DIAGNOSTIC TESTS

Dengue: Data, Maps and Statistics." Pan American Health Organization, March 2023.

Dengue: Global Alert Response GAR." World Health Organization, 1998.

Di Sabatino, David, et al. "West Nile Virus: Biology, Transmission, and Human Infection." *Clinical Microbiology Reviews*, vol. 25, no. 4, 2014.

Dietzschold et al., "Characterization of the L Gene and 5' Trailer Region of Ebola Virus." *Journal of General Virology*, vol. 86, no. 5, 2005

Dietzschold, Bernhard, et al. "Characterization of an Attenuated Rabies Virus Mutant: Potential for Use in Wildlife Oral Vaccination." *Journal of Virology*, vol. 75, no. 2, 2001.

Farquhar and Palade, "Junctional Complexes in Various Epithelia," *The Journal of Cell Biology*, 2023.

Finnegan, Christopher J., et al. "Rabies." *Pediatric Emergency Care*, vol. 18, no. 4, 2002

Furuse et al., "Occludin: A Novel Integral Membrane Protein Localizing at Tight Junctions," *The Journal of Cell Biology*, 1993.

Garg et al., "Neuroimaging in Dengue Virus-Associated Encephalitis," *Neurology India*, 2017.

Gloor, Sergio M., et al. "Molecular and cellular permeability control at the blood–brain barrier." *Brain research reviews* 36.2-3 (2020): 258-264.

Gralinski, Lisa E., et al. "MAV-1 Infection of Endothelial Cells Alters Tight Junction Protein Expression and Disrupts the Blood-Brain Barrier." *Virology*, vol. 393, no. 1, 2009.

Green et al., "Dengue Virus-Mediated Endothelial Cell Activation and Dysfunction: Role of MMP," *Journal of Virology*, 2023.

Gubler D.J. Dengue/dengue haemorrhagic fever: history and current status. *Novartis Found Symp.* 2006;277:3–16.

Gubler D.J. Epidemic dengue/dengue hemorrhagic fever as a public health, social and economic problem in the 21st century. *Trends Microbiol.* 2002;10:100–103.

Gubler, D.J., 2014. Dengue and Dengue Hemorrhagic Fever. In: Gubler, D.J., Ooi, E.E., Vasudevan, S., Farrar, J. (Eds.), 2nd edn. CAB International, 1–29.

Gubler, Duane J. "Dengue and Dengue Hemorrhagic Fever." *Clinical Microbiology Reviews*, vol. 11, no. 3, 1998.

Gubler, Duane J. "The Global Emergence/Resurgence of Arboviral Diseases as Public Health Problems." *Archives of Medical Research*, vol. 33, no. 4, 2002.

Gupta et al., "Therapeutic and Preventive Options for the Management of Dengue and Severe Dengue," *Expert Review of Anti-infective Therapy*, 2013.

Guy B., Briand O., Lang J., Saville M., Jackson N. Development of the Sanofi Pasteur tetravalent dengue vaccine: One more step forward. *Vaccine*. 2015;33:7100–7111.

Guzman A., Istúriz R.E. Update on the global spread of dengue. *Int. J. Antimicrob. Agents*. 2010;36:S40–S42.

Guzman, Maria G., and Istúriz, R. Eduardo. "Update on the Global Spread of Dengue." *International Journal of Antimicrobial Agents*, vol. 36, no. 1, 2010.

Hadinegoro S.R., Arredondo-García J.L., Capeding M.R., Deseda C., Chotpitayasunondh T., Dietze R. Efficacy and long-term safety of a dengue vaccine in regions of endemic disease. *N. Engl. J. Med*. 2015;373:1195–1206.

Hagendorf, N. & Conzelmann, K.-K. Recombinant fluorescent rabies virus vectors for tracing neurons and synaptic connections. *Cold Spring Harb. Protoc*. <http://dx.doi.org/10.1101/pdb.top089391> (2015).

Hagendorf, N. & Conzelmann, K.-K. Recombinant fluorescent rabies virus vectors for tracing neurons and synaptic connections. *Cold Spring Harb. Protoc*. <http://dx.doi.org/10.1101/pdb.top089391> (2015).

Halstead et al., "In Vivo Enhancement of Dengue Virus Infection in Rhesus Monkeys by Passively Transferred Antibody," *Journal of Infectious Diseases*, 2022.

Hang V.T., Nguyet N.M., Trung D.T., Tricou V., Yoksan S., Dung N.M. Diagnostic accuracy of NS1 ELISA and lateral flow rapid tests for dengue sensitivity, specificity and relationship to viraemia and antibody responses. *PloS Negl. Trop. Dis*. 2009;3

Henchal E.A., Gentry M.K., McCown J.M., Brandt W.E. Dengue virus-specific and flavivirus group determinants identified with monoclonal antibodies by indirect immunofluorescence. *Am. J. Trop. Med. Hyg*. 1982;31:830–836.

Hojjatie, Sara L., et al. "Vector-Borne Infections." *Emerging Ocular Infections*. Cham: Springer International Publishing, 2023. 79-90.

Huhtamo, Eili, et al. "Emerging Flaviviruses in Europe: A Call for Awareness." *Expert Review of Anti-infective Therapy*, vol. 12, no. 8, 2014.

Hunsperger E.A. Evaluation of commercially available anti-dengue virus immunoglobulin M tests. *Emerg. Infect. Dis.* 2009;15:436–440.

Irfan A.R., Sanjay K., Vivek K.B., Jeongheui L., Yong-Ha P. Prevention and control strategies to counter ZIKA epidemic. *Front. Microbiol.* 2017;8:305.

Irfan, A.R., Jameel, B.L., Vivek, K.B., Yong-Ha, Park, 2017a. Irfan A Rather, Jameel B Lone, Vivek K Bajpai, Yong-Ha Park. Zika virus infection during pregnancy and congenital abnormalities. *Front. Microbiol.* 8; 581.

Irfan, Muhammad, et al. "A Review on the Epidemiology and Clinical Management of Dengue Infection in Pakistan." *Journal of Pakistan Medical Association*, vol. 67, no. 8, 2017.

Islam R, Parvez MS, Anwar S, Hosen MJ. Delineating blueprint of an epitope-based peptide vaccine against the multiple serovars of dengue virus: A hierarchical reverse vaccinology approach. *Informatics in Medicine Unlocked*. 2020 Jan 1; 20:100430.

Iwasaki, Y., Ohtani, S. & Clark, H. F. Maturation of rabies virus by budding from neuronal cell membrane in suckling mouse brain. *J. Virol.* 15, 1020–1023 (1975).

Jackson, "The Pathogenesis of Rabies and Other Lyssaviral Infections: Recent Studies," *Current Topics in Microbiology and Immunology*, 2002.

Jackson, Alan C. "Rabies: A Review." *Canadian Journal of Neurological Sciences*, vol. 27, no. 4, 2000.

Jan et al., "Dengue Virus-Induced Neuronal Apoptosis," *Neuroreport*, 2000.

Jessie et al., "Localization of Dengue Virus in Naturally Infected Human Tissues, by Immunohistochemistry and In Situ Hybridization," *Journal of Infectious Diseases*, 2004.

- Junjhon J., Pennington J.G., Edwards T.J., Perera R., Lanman J., Kuhn R.J. Ultrastructural characterization and three-dimensional architecture of replication sites in dengue virus-infected mosquito cells. *J. Virol.* 2014; 88:4687–4697.
- Kalita and Misra, "Comparison of EEG Changes in Japanese Encephalitis and Dengue," *Journal of the Neurological Sciences*, 2006.
- Kanesa-Than, N., Sun, W., Kim-Ahn, G., Van-Albert, S., Putnak, J., King, A., Raengsakulrach, B., Christ-Schmidt, H., Gilson, K., Zahradnik, J.M., Vaughn, D.W., Innis, B., Saluzzo, J.F., Hoker-Jr, Ch., 2001. Safety and immunogenicity of attenuated dengue virus vaccines (Aventis Pasteur) in human volunteers. *Vaccine* 19, 3179–3188.
- Khursheed, M., Khan, U.R., Ejaz, K., Fayyaz, J., Qamar, I., Razzak, J.A., 2013. A comparison of WHO guidelines issues in 1997 and 2009 for dengue fever- single center experience. *J. Park Med. Assoc.* 36, 670–674.
- Khursheed, Muhammad, et al. "Dengue Fever Treatment with Carica Papaya Leaves Extracts." *Asian Pacific Journal of Tropical Biomedicine*, vol. 3, no. 8, 2013.
- Kitchener S., Nissen M., Nasveld P., Forrat R., Yoksan S., Lang J., Francois-Saluzzo J. Immunogenicity and safety of two live-attenuated tetravalent dengue vaccine formulations in healthy Australian adults. *Vaccine*. 2006;24:1238–1241.
- Knapik, S., Sigrist, S., & Tanimoto, H. (2011). Bruchpilot, a synaptic active zone protein for anesthesia-resistant memory. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 31(9), 3453–3458. <https://doi.org/10.1523/JNEUROSCI.2585-10.2011>
- Knobel, Darryn L., et al. "Re-Evaluating the Burden of Rabies in Africa and Asia." *Bulletin of the World Health Organization*, vol. 83, no. 5, 2005.
- Koyuncu, O. O., Hogue, I. B. & Enquist, L. W. Virus infections in the nervous system. *Cell Host Microbe* 13, 379–393 (2013).

Kuhn R.J., Zhang W., Rossmann M.G., Pletnev S.V., Corver J., Lenches E., Jones C.T., Mukhopadhyay S., Chipman P.R., Strauss E.G., Baker T.S., Strauss J.H. Structure of dengue virus: implications for flavivirus organization, maturation, and fusion. *Cell*. 2002;108:717–725.

Kumar et al., "Rabies Epidemiology and Control in India: A Review," *Veterinary World*, 2017.

Lanciotti R.S., Calisher C.H., Gubler D.J., Chang G.J., Vorndam A.V. Rapid detection and typing of dengue viruses from clinical samples by using Reverse Transcriptase Polymerase Chain Reaction. *J. Clin. Microbiol.* 1992;30:545–551.

Lehner, Michael D., et al. "Functional and Structural Integrity of the Blood-Brain Barrier in Mice after Treatment with a Novel TLR4 Agonist Derived from *Neisseria Meningitidis*." *Journal of Neuroinflammation*, vol. 8, no. 1, 2011.

Libbey, J. & Fujinami, R. S. Adaptive immune response to viral infections in the central nervous system. *Hanb. Clin. Neurol.* 123, 225–247 (2014).

Limonta et al., "Dengue Virus Infection of Human Endothelial Cells Leads to Chemokine Production, Complement Activation, and Apoptosis," *Journal of Immunology*, 1998.

Lin, Chien-Chen, et al. "Dengue Virus Infection Downregulates Matrix Metalloproteinase-9 Expression in Human Endothelial Cells through Toll-Like Receptor 3." *Journal of Virology* 84.11 (2010): 5971-5979.

Lin, Chien-Chen, et al. "Dengue Virus Infection Downregulates Tumor Necrosis Factor- α Production in Human Endothelial Cells through Toll-Like Receptor 3." *Journal of Immunology* 182.6 (2009): 3677-3685.

Lin, Chien-Chen, et al. "Dengue Virus Infection Induces IL-18 Production in Human Endothelial Cells through Toll-Like Receptor 3." *Journal of Immunology* 177.12 (2006): 8551-8559.

- Liou et al., "Dengue Fever with Encephalopathy: EEG and MRI Findings," *Pediatric Neurology*, 2008.
- Lobigs, M., Mullbacher, A., Wang, Y., Pavy, M. & Lee, E. Role of type I and type II interferon responses in recovery from infection with an encephalitic flavivirus. *J. Gen. Virol.* 84, 567–572 (2003).
- Madi et al., "Study of Clinical Profile and Outcome of Dengue Fever in a Tertiary Care Hospital in Semi Urban South India," *Indian Journal of Public Health Research & Development*, 2014.
- Maghazachi, Azzam A. "Role of chemokines in the biology of natural killer cells." *The Chemokine System in Experimental and Clinical Hematology* (2010): 37-58.
- Mamdouh et al., "Dengue Meningitis: A Rare Manifestation of Dengue Infection," *Annals of Indian Academy of Neurology*, 2013.
- Martin-Padura et al., "Junctional Adhesion Molecule: A Novel Member of the Immunoglobulin Superfamily of Cell Adhesion Molecules," *The Journal of Cell Biology*, 1998.
- Martinez L.J., Lin L., Blaylock J.M., Lyons A.G., Bauer K.M., De La Barrera R. Safety and Immunogenicity of a Dengue Virus Serotype-1 Purified -Inactivated Vaccine: Results of a Phase 1 Clinical Trial. *Am. J. Trop Med. Hyg.* 2015;93:454–460.
- Mathew et al., "Dengue Encephalitis- A Diagnostic Challenge," *Journal of Clinical and Diagnostic Research*, 2015.
- McCallum, "Dengue Fever: The Global Spread of a Mosquito-Borne Disease." *Environmental History*, vol. 13, no. 4, 2008.
- McCallum, J.E., 2008. *Military medicine from ancient times to the 21st century: Dengue fever* ABC-CLIO, Inc., California, USA.

- McGavern, D. B. & Kang, S. S. Illuminating viral infections in the nervous system. *Nature* 11, 318–329 (2011).
- Mishra and Basu, "Japanese Encephalitis Virus: An Emerging Global Pathogen," *Journal of Biosciences*, 2008.
- Misra et al., "Dengue-associated Transient Muscular Dysfunction: A Prospective Study of 50 Patients," *Annals of Indian Academy of Neurology*, 2006.
- Misra et al., "Dengue-associated Transient Muscular Dysfunction: A Prospective Study of 50 Patients," *Annals of Indian Academy of Neurology*, 2015.
- Misra et al., "Dengue-associated Transient Muscular Dysfunction: A Prospective Study of 50 Patients," *Annals of Indian Academy of Neurology*, 2015.
- Mujtaba A.B., Safikur R., Irfan A.R., Iqbal B., Shafiya S., Hamira K., Mohammad A.K., Rinki M., Arif T.J. Coronavirus Disease-2019 (COVID-19) in 2020: A perspective study of a global pandemic. *Curr. Pharm. Des.* 2021 doi: 10.2174/1381612826666201118112912.
- Mujtaba, Ghulam, et al. "Dengue Fever: Causes, Complications, and Vaccine Strategies." *Journal of Immunology Research*, vol. 2021, 2021.
- Mukhopadhyay S., Kim B.S., Chipman P.R., Rossmann M.G., Kuhn R.J. Structure of West Nile virus. *Science*. 2003;302:5643248.
- Mukhopadhyay S., Kuhn R.J., Rossmann M.G. A structural perspective of the Flavivirus life cycle. *Nat. Rev. Microbiol.* 2005;3:13–22.
- Murphy, Frederick A. "Japanese Encephalitis." In "Fields Virology," edited by David M. Knipe and Peter M. Howley, 4th ed., vol. 1, 1999.
- Neal, Jonathan W. "Flaviviruses Are Neurotropic, but How Do They Invade the CNS?" *The Journal of Infection*, vol. 69, no. 3, 2014.
- Nel, Louis H., et al. "Rabies in the Developing World: Challenges and Control." *Onderstepoort Journal of Veterinary Research*, vol. 80, no. 1, 2013.

Osorio, J.E., Velez, I.D., Omson, C., Thomson, C., a Lopez, L., Jimenez, A., Haller, A.A., Silengo, S., Scott, J., Boroughs, K.L., 2014. Safety and immunogenicity of a recombinant live attenuated tetravalent dengue vaccine (DENVax) in avivirus-naive healthy adults in Colombia: a randomised, placebo-controlled, phase 1 study. e Lancet Infectious Diseases 14, 830–838.

Pagni S., Fernandez-Sesma A. Evasion of the human innate immune system by dengue virus. Immunol. Res. 2012;54:152–159.

Panchal R, Bapat S, Mukherjee S, Chowdhary A. *In silico* binding analysis of lutein and rosmarinic acid against envelope domain III protein of dengue virus. Indian J Pharmacol. 2021 Nov-Dec;53(6):471-479. doi: 10.4103/ijp.IJP_576_19. PMID: 34975135; PMCID: PMC8764985.

Pancharoen and Thisyakorn, "Neurological Manifestations in Dengue Patients," Southeast Asian Journal of Tropical Medicine and Public Health, 2001.

Paweska, Janusz T., et al. "Rabies Virus." Veterinary Clinics: Small Animal Practice, vol. 36, no. 4, 2006.

Potokar, Maja, et al. "Astrocytes and Microglia in Acute Encephalitis." Neuropathology and Applied Neurobiology, vol. 40, no. 1, 2014.

Pulzova, Lucia, et al. "Modeling the Blood-Brain Barrier: Beyond the Endothelial Cells Front." Immunology, vol. 10, 2009.

Putnak J.R., Collier B.A., Voss G., Vaughn D.W., Clements D., Peters I., Bignami G., Houngh U., Chen R.C., Barvir D.A., Seriwatana J., Cayphas S., Garçon N., Gheysen D., Kanesthasan N., McDonell M., Humphreys T., Eckels K.H., Prieels J.P., Innis B.L. An evaluation of dengue type-2 inactivated, recombinant subunit, and live-attenuated vaccine candidates in the rhesus macaque model. Vaccine. 2005;23:4442–4452.

Putnak, P., Barvir, D.A.J., Burrous, M., Dubois, D.R., D'Andrea, V.M., Hoke, C.H., Sadoff, J.C., Eckels, K.H., 1996. Development of a purified, inactivated, dengue-2 virus vaccine prototype in Vero cells: immunogenicity and protection in mice and rhesus monkeys. *J. Infect. Dis.* 174, 1176–1184.

Rather I.A., Parray H.A., Lone J.B., Paek W.K., Lim J., Bajpai V.K., Park Y.H. Prevention and control strategies to counter dengue virus infection. *Front. Cell Infect. Microbiol.* 2017;7:336.

Raviprakash K., Wang D., Ewing D., Holman D.H., Block K., Woraratanadharm J., Chen L., Hayes C., Dong J.Y., Porter K. A tetravalent dengue vaccine based on a complex adenovirus vector provides significant protection in rhesus monkeys against all four serotypes of dengue virus. *J. Virol.* 2008;82:6927–6934.

Rodenhuis-Zybert et al., "Dengue Virus Life Cycle: Viral and Host Factors Modulating Infectivity," *Cellular and Molecular Life Sciences*, 2010.

Roy, Anirban, et al. "Mechanisms of Rabies Virus-Induced Neuronal Dysfunction and Death." *PLOS Neglected Tropical Diseases*, vol. 9, no. 7, 2015.

Rupprecht, Charles E., et al. "Rabies Transmission to Humans." *Clinical Infectious Diseases*, vol. 35, no. 7, 2002.

Ruzek, Daniel, et al. "Tick-Borne Encephalitis: Pathogenesis and Clinical Implications." *Travel Medicine and Infectious Disease*, vol. 9, no. 4, 2011.

Sahu et al., "Neurological Complications of Dengue Fever: Experience from a Tertiary Center of North India," *Annals of Indian Academy of Neurology*, 2014.

Salazar M.I., Richardson J.H., Sánchez-Vargas I., Olson K.E., Beaty B.J. How the dengue virus replicates in infected cells. *BMC Microbiol.* 2007;7:1–12.

Sarkar B, Ullah MA, Araf Y. A systematic and reverse vaccinology approach to design novel subunit vaccines against Dengue virus type-1 (DENV-1) and human Papillomavirus-16 (HPV-16). *Informatics in Medicine Unlocked*. 2020 Jan 1; 19:100343.

Schnell, Matthias J., et al. "Pathogenesis of Rabies." *Current Topics in Microbiology and Immunology*, vol. 339, 2010.

Shill DK, Jahan S, Alam MM, Limon MB, Alam M, Rahman MZ, Rahman M. S-Adenosyl-l-Homocysteine Exhibits Potential Antiviral Activity Against Dengue Virus Serotype-3 (DENV-3) in Bangladesh: A Viroinformatics-Based Approach. *Bioinformatics and Biology Insights*. 2023 Feb; 17:11779322231158249.

Shresta et al., "Critical Role of Macrophages in Dengue Virus-Induced Endothelial Cell Injury and Vascular Leakage," *Journal of Virology*, 2012.

Shrivastava, S., Tiraki, D., Diwan, A., Lalwani, S.K., Modak, M., Mishra, A.C. and Arankalle, V.A., 2018. Co-circulation of all the four dengue virus serotypes and detection of a novel clade of DENV-4 (genotype I) virus in Pune, India during 2016 season PLoS One. 13, 1-19.

Siler J.F., Hall M.W., Hitchens A.P. Dengue: its history, epidemiology, mechanism of transmission, etiology, clinical manifestations, immunity and prevention. *Philippine J. Sci*. 1926;29:1–304.

Simmonds P., Becher P., Bukh J. ICTV Virus Taxonomy Profile: Flaviviridae. *J. Gen. Virol*. 2017;98:2–3.

Soares and Marzia, "Dengue: Neurological Manifestations and Other Clinical Concerns," *The Lancet Infectious Diseases*, 2014.

Soares et al., "Dengue Infection: Neurological Manifestations and Cerebrospinal Fluid (CSF) Analysis," *Journal of the Neurological Sciences*, 2011.

Soares et al., "Dengue Infection: Neurological Manifestations and Cerebrospinal Fluid (CSF) Analysis," *Journal of the Neurological Sciences*, 2006.

Soares et al., "Dengue Virus Infection Associated with Nervous System Manifestations," *Clinical Infectious Diseases*, 2010.

Solbrig and Perng, "Neurological Manifestations of Dengue Infection," *Frontiers in Cellular and Infection Microbiology*, 2015.

Solomon, Tom, et al. "Flavivirus Encephalitis." *New England Journal of Medicine*, vol. 351, no. 4, 2004.

Srikiatkachorn et al., "Natural History of Plasma Leakage in Dengue Hemorrhagic Fever: A Serial Ultrasonographic Study," *Pediatric Infectious Disease Journal*, 2007.

Stadler K., Allison S.L., Schlich J., Heinz F.X. Proteolytic activation of tick-borne encephalitis virus by furin. *J. Virol.* 1997;71:8475–8481.

Su, Hui-Ling, et al. "Dengue Virus Infection Downregulates IL-6 Production in Human Endothelial Cells through Toll-Like Receptor 3." *Journal of Virology* 82.10 (2008): 4773-4782.

Su, Hui-Ling, et al. "Dengue Virus Infection Downregulates Occludin Expression in Human Endothelial Cells through Toll-Like Receptor 3." *Journal of Virology* 81.13 (2007): 7116-7124.

Suen, William W., et al. "Mechanisms of West Nile Virus Transmission and Pathogenesis." *Trends in Microbiology*, vol. 22, no. 9, 2014.

Suthar et al., "Dengue Virus Infection and Host Response in the Nervous System," *Reviews in Medical Virology*, 2013.

Tseng, Chia-Yu, et al. "Dengue Virus Infection Induces Nitric Oxide Production through Inducible Nitric Oxide Synthase Pathway in Human Endothelial Cells." *Journal of Biomedical Science* 18.1 (2011): 1-13.

Tsukita and Furuse, "Claudin-based Barrier in Simple and Stratified Cellular Sheets," *Current Opinion in Cell Biology*, 2002.

- Tsukita et al., "Structural and Signaling Molecules Come Together at Tight Junctions," Cold Spring Harbor Perspectives in Biology, 2009.
- Tuiskunen Bäck, A., and Lundkvist, Åke. "Dengue Viruses - An Overview." *Infection Ecology & Epidemiology*, vol. 3, 2012.
- Tuiskunen-B A.N., Lundkvist A. Dengue viruses an overview. *Infect. Ecol. Epidemiol.* 2012;3:19839.
- Turtle, Lance, et al. "Human T Cell Responses to Japanese Encephalitis Virus in Health and Disease." *Frontiers in Immunology*, vol. 7, 2012.
- Van den Pol, A. N., Ding, S. & Robek, M. D. Long distance interferon signaling within the brain blocks virus spread. *J. Virol.*88, 3695–3704 (2014).
- Vorndam V., Kuno G., Gubler D.J., Kuno G. CAB International. Laboratory diagnosis of dengue virus infections; New York: 1997. Dengue and dengue hemorrhagic fever; pp. 313–333.
- Wadood A., Mehmood A., Khan H., Ilyas M., Ahmad A., Alarjah M., Abu-Izneid T. Epitopes based drug design for dengue virus envelope protein: A computational approach. *Comput. Biol. Chem.* 2017;71:152–160.
- Wagh DA, Rasse TM, Asan E, Hofbauer A, Schwenkert I, Dürrbeck H, Buchner S, Dabauvalle MC, Schmidt M, Qin G, Wichmann C, Kittel R, Sigrist SJ, Buchner E. Bruchpilot, a protein with homology to ELKS/CAST, is required for structural integrity and function of synaptic active zones in *Drosophila*. *Neuron*. 2006 Mar 16;49(6):833-44. doi: 10.1016/j.neuron.2006.02.008. Erratum in: *Neuron*. 2006 Jul 20;51(2):275. PMID: 16543132.
- Wakim, L. M., Woodward-Davis, A. & Bevan, M. J. Memory T cells persisting within the brain after local infection show functional adaptations to their tissue of residence. *Proc. Natl Acad. Sci. USA* 107, 17872–17879 (2010).

Wang et al., "Japanese Encephalitis Virus Induces Human Neural Stem/Progenitor Cells to Secrete Proinflammatory Cytokines," *Journal of Neuroinflammation*, 2012.

Wang, T. et al. Toll-like receptor 3 mediates West Nile virus entry into the brain causing lethal encephalitis. *Nat. Med.* 10, 1366–1373 (2004).

Wang, Wen-Ling, et al. "Dengue Virus Infection Upregulates Matrix Metalloproteinase-2 Expression in Human Endothelial Cells through Toll-Like Receptor 3." *Journal of Virology* 82.24 (2008): 11712-11720.

Welsch S., Miller S., Romero-Brey I., Merz A., Bleck C.K., Walther P., Fuller S.D., Antony C., Krijnse-Locker J., Bartenschlager R. Composition and three-dimensional architecture of the dengue virus replication and assembly sites. *Cell Host Microbe*. 2009;5:365–375.

World Health Organization (WHO), 1997. Dengue haemorrhagic fever: diagnosis, treatment, prevention and control, 2nd ed. Geneva WHO. World Health Organization (WHO), 2021. History and Origin of Dengue Virus.

World Health Organization, "Dengue Haemorrhagic Fever: Diagnosis, Treatment, Prevention and Control," 1997.

Young P.R., Hilditch P.A., Bletchly C., Halloran W. An antigen capture enzyme-linked immunosorbent assay reveals high levels of the dengue virus protein NS1 in the sera of infected patients. *J. Clin. Microbiol.* 2000;38:1053–1057.

Yu I.M., Zhang W., Holdaway H.A., Li L., Kostyuchenko V.A., Chipman P.R., Kuhn R.J., Rossmann M.G., Chen J. Structure of the immature dengue virus at low pH primes proteolytic maturation. *Science*. 2008;319:1834–1837.

Zaichick, S. V. et al. The herpesvirus VP1/2 Protein is an effector of dynein-mediated capsid transport and neuroinvasion. *Cell Host Microbe* 13, 193–203 (2013) Podolsky, M. A. et al.

Zellweger R., Miller R., Eddy W., White L., Johnston R., Shresta S. Role of Humoral versus Cellular Responses Induced by a Protective Dengue Vaccine Candidate. *PLoS Pathogens*. 2013;9.

Zhang, Shuxian, et al. "Japanese Encephalitis Virus Infects Neural Progenitor Cells and Decreases Their Proliferation." *Journal of Neuroimmunology*, vol. 267, no. 1-2, 2014.

Zlokovic, Berislav V. "The blood-brain barrier in health and chronic neurodegenerative disorders." *Neuron* 57.2 (2008): 178-201.

TurnitinUK Originality Report

Processed on: 05-Dec-2023 10:39 AM GMT

ID: 217909785

Word Count: 28378

Submitted: 1

RE-2022-189001-plag-report By Research Experts

- Turnitin Report

Similarity Index

8%

Similarity by Source

Internet Sources:	4%
Publications:	5%
Student Papers:	1%

1% match (Guo-Hong Li, Zhi-Jie Ning, Yi-Ming Liu, Xiao-Hong Li. "Neurological Manifestations of Dengue Infection", *Frontiers in Cellular and Infection Microbiology*, 2017)
[Guo-Hong Li, Zhi-Jie Ning, Yi-Ming Liu, Xiao-Hong Li. "Neurological Manifestations of Dengue Infection", *Frontiers in Cellular and Infection Microbiology*, 2017](#)

1% match (Internet from 02-Oct-2022)
http://personal.uvlf.sk/personal-pages/mangesh.bhide/share/publications/Michalicova_2018.pdf

< 1% match (Internet from 20-Jun-2023)
<https://www.science.gov/topicpages/t/toil-like+receptors+expression>

< 1% match (Internet from 03-Jul-2023)
<https://www.science.gov/topicpages/p/proinflammatory+cytokine+expression>

< 1% match (Internet from 08-Jun-2023)
<https://www.science.gov/topicpages/a/alphavirus+replicons+expressing>

< 1% match (Internet from 05-Jul-2023)
<https://www.science.gov/topicpages/v/virus-derived+retroviral+vectors>

< 1% match (Internet from 20-Jul-2023)
<https://www.science.gov/topicpages/p/peripheral+lipopolysaccharide+induce>

< 1% match (Maria-Angélica Calderón-Peláez, Myriam L. Velandia-Romero, Leidy Y. Bastidas-Legarda, Edgar O. Beltrán et al. "Dengue Virus Infection of Blood-Brain Barrier Cells: Consequences of Severe Disease", *Frontiers in Microbiology*, 2019)
[Maria-Angélica Calderón-Peláez, Myriam L. Velandia-Romero, Leidy Y. Bastidas-Legarda, Edgar O. Beltrán et al. "Dengue Virus Infection of Blood-Brain Barrier Cells: Consequences of Severe Disease", *Frontiers in Microbiology*, 2019](#)

< 1% match (Simin Lu, Jianye Wang, Zhangping He, Siqin He, Kang Zheng, Man Xu, Shuai Yuan, Yimou Wu. "Treponema pallidum Tp0751 alters the expression of tight junction proteins by promoting bEnd3 cell apoptosis and IL-6 secretion", *International Journal of Medical Microbiology*, 2022)
[Simin Lu, Jianye Wang, Zhangping He, Siqin He, Kang Zheng, Man Xu, Shuai Yuan, Yimou Wu. "Treponema pallidum Tp0751 alters the expression of tight junction proteins by promoting bEnd3 cell apoptosis and IL-6 secretion", *International Journal of Medical Microbiology*, 2022](#)

< 1% match (Internet from 05-Aug-2023)
https://d.docksci.com/tight-junctions-go-viral_5a2cd30ed64ab2f6cd4e60a7.html

< 1% match (Methods in Molecular Biology, 2016.)
[Methods in Molecular Biology, 2016.](#)

< 1% match (Rushil S. Bhatt, Sweta T. Kothari, Devanshi J. Gohil, Marsha D'Souza, Abhay S. Chowdhary. "Novel evidence of microglial immune response in impairment of Dengue infection of CNS", *Immunobiology*, 2015)
[Rushil S. Bhatt, Sweta T. Kothari, Devanshi J. Gohil, Marsha D'Souza, Abhay S. Chowdhary. "Novel evidence of microglial immune response in impairment of Dengue infection of CNS", *Immunobiology*, 2015](#)

< 1% match (Internet from 26-May-2023)
<https://WWW.MDPI.COM/1999-4915/12/4/405>

< 1% match (Internet from 21-Feb-2020)
<https://www.mdpi.com/1420-3049/23/11/2848/htm>

< 1% match (Internet from 05-Oct-2023)
<https://WWW.MDPI.COM/2072-6643/15/6/1869>

< 1% match (Internet from 09-Oct-2023)
<https://www.medrxiv.org/content/10.1101/2023.07.17.23292782v2.full>

< 1% match (Internet from 04-Dec-2023)
<https://www.medrxiv.org/content/10.1101/2022.05.17.22275235v2.full.pdf>

< 1% match ("Stem Cells and Good Manufacturing Practices", Springer Science and Business Media LLC, 2021)
["Stem Cells and Good Manufacturing Practices", Springer Science and Business Media LLC, 2021](#)

< 1% match (Yan Wen Dai, Chang Cheng Zhang, Hai Xia Zhao, Jing Zhi Wan et al. "Chikusetsusaponin V attenuates lipopolysaccharide-induced liver injury in mice", *Immunopharmacology and Immunotoxicology*, 2016)
[Yan Wen Dai, Chang Cheng Zhang, Hai Xia Zhao, Jing Zhi Wan et al. "Chikusetsusaponin V attenuates lipopolysaccharide-induced liver injury in mice", *Immunopharmacology and Immunotoxicology*, 2016](#)

< 1% match ()
["Abstracts from the International Science Symposium on HIV and Infectious Diseases \(ISSHID 2019\): Infectious diseases", BMC Infectious Diseases](#)

< 1% match (Internet from 04-Mar-2015)
<http://www.ncbi.nlm.nih.gov/gene/773>

< 1% match ()

Lucas de Freitas Pedrosa, Paul de Vos, João Paulo Fabi. "Nature's soothing solution: Harnessing the potential of food-derived polysaccharides to control inflammation", *Current Research in Structural Biology*

< 1% match (Internet from 21-Jul-2020)
https://mafiaadoc.com/Flavivirus-encephalitis_5cb80d73097c47eb4c8b4573.html

< 1% match (Internet from 11-Oct-2023)
<https://worldwidescience.org/topicpages/s/secondary+dengue+infection.html>

< 1% match (Internet from 03-Dec-2023)
<https://worldwidescience.org/topicpages/s/suppressed+lips-induced+nos-2.html>

< 1% match (Internet from 11-Oct-2023)
<https://worldwidescience.org/topicpages/s/serine+protease+proteinase.html>

< 1% match (Internet from 28-Sep-2021)
<https://academic.oup.com/jid/article/223/10/1766/5906645>

< 1% match (Mernan Jamal Sabir, Najla Bint Saud Al-Saud, Sabah Mohmoud Hassan. "Dengue and human health: A global scenario of its occurrence, diagnosis and therapeutics", *Saudi Journal of Biological Sciences*, 2021)
Mernan Jamal Sabir, Najla Bint Saud Al-Saud, Sabah Mohmoud Hassan. "Dengue and human health: A global scenario of its occurrence, diagnosis and therapeutics", *Saudi Journal of Biological Sciences*, 2021

< 1% match (Kiran Bala Sharma, Sudhanshu Vrat, Manjula Kalia. "Pathobiology of Japanese encephalitis virus infection", *Molecular Aspects of Medicine*, 2021)
Kiran Bala Sharma, Sudhanshu Vrat, Manjula Kalia. "Pathobiology of Japanese encephalitis virus infection", *Molecular Aspects of Medicine*, 2021

< 1% match (Tariq Ahmad Baig, Haroon M. Haniffa, Hina Siddiqui, Syeda Farah Shah, Almas Jabeen. "A new acyl derivative of sulfadimethoxine inhibits phagocyte oxidative burst and ameliorates inflammation in a mice model of zymosan-induced generalised inflammation", *Inflammopharmacology*, 2023)
Tariq Ahmad Baig, Haroon M. Haniffa, Hina Siddiqui, Syeda Farah Shah, Almas Jabeen. "A new acyl derivative of sulfadimethoxine inhibits phagocyte oxidative burst and ameliorates inflammation in a mice model of zymosan-induced generalised inflammation", *Inflammopharmacology*, 2023

< 1% match ("Neglected Tropical Diseases and Conditions of the Nervous System", Springer Nature, 2014)
"Neglected Tropical Diseases and Conditions of the Nervous System", Springer Nature, 2014

< 1% match (Pu, H.. "Limited role of COX-2 in HIV Tat-induced alterations of tight junction protein expression and disruption of the blood-brain barrier", *Brain Research*, 20071212)
Pu, H.. "Limited role of COX-2 in HIV Tat-induced alterations of tight junction protein expression and disruption of the blood-brain barrier", *Brain Research*, 20071212

< 1% match (Carlo Amarin Daep, Jorge L. Muñoz-Jordán, Eliseo Alberto Eugenin. "Flaviviruses, an expanding threat in public health: focus on dengue, West Nile, and Japanese encephalitis virus", *Journal of NeuroVirology*, 2014)
Carlo Amarin Daep, Jorge L. Muñoz-Jordán, Eliseo Alberto Eugenin. "Flaviviruses, an expanding threat in public health: focus on dengue, West Nile, and Japanese encephalitis virus", *Journal of NeuroVirology*, 2014

< 1% match (student papers from 15-Apr-2016)
Submitted to Liverpool John Moores University on 2016-04-15

< 1% match (student papers from 28-Mar-2012)
Submitted to Liverpool John Moores University on 2012-03-28

< 1% match (Internet from 20-May-2023)
<https://www.biorxiv.org/content/10.1101/2023.03.08.531740v1.full>

< 1% match (Internet from 05-Oct-2022)
<https://www.biorxiv.org/content/10.1101/2022.07.17.500327v1.full.pdf>

< 1% match (Florencia B. González, Silvina R. Villar, Rodrigo Fernández Bussy, Gaëlle H. Martin et al. "Immunoendocrine dysbalance during uncontrolled T. cruzi infection is associated with the acquisition of a Th-1-like phenotype by Foxp3+ T cells", *Brain, Behavior, and Immunity*, 2015)
Florencia B. González, Silvina R. Villar, Rodrigo Fernández Bussy, Gaëlle H. Martin et al. "Immunoendocrine dysbalance during uncontrolled T. cruzi infection is associated with the acquisition of a Th-1-like phenotype by Foxp3+ T cells", *Brain, Behavior, and Immunity*, 2015

< 1% match (Internet from 04-Oct-2021)
<https://pure.uva.nl/ws/files/39895600/Thesis.pdf>

< 1% match (Internet from 03-Dec-2017)
http://www.nature.com/articles/nmeth.3250?code=c5da584f-ddd6-4f15-b63c-ef54243670c9&error=cookies_not_supported

< 1% match (Internet from 01-Aug-2023)
https://www.nature.com/articles/ncomms8489?code=32e25be7-5ac9-45d9-a9d7-d9452b421bfd&error=cookies_not_supported

< 1% match ()
Grunnill, Martin David. "Inapparent and Vertically Transmitted Infections in Two Host-Virus Systems", *Collage of Life and Environmental Sciences*, 2016

< 1% match ("High-Resolution Imaging of Cellular Proteins", Springer Science and Business Media LLC, 2016)
"High-Resolution Imaging of Cellular Proteins", Springer Science and Business Media LLC, 2016

< 1% match (student papers from 16-Jun-2022)
Submitted to The University of Manchester on 2022-06-16

< 1% match (student papers from 06-May-2014)
Submitted to Cardiff University on 2014-05-06

< 1% match (student papers from 24-Mar-2023)
Submitted to Western Illinois University on 2023-03-24

< 1% match (Internet from 05-Dec-2022)
https://mdpi-res.com/bookfiles/book/6275/Antioxidants_in_AgeRelated_Diseases_and_AntiAging_Strategies.pdf?v=1666993682

< 1% match (Internet from 21-Apr-2023)
<https://moam.info/development-of-multiplex-real-time-rt-pcr-for-the-5b7c988b097c47412c8b46b6.html>

< 1% match (Internet from 25-May-2020)
<https://www.neb.sg/protocols/2016/11/08/luna-universal-qpcr-master-mix-protocol-m3003>

< 1% match (student papers from 01-Mar-2022)
 Submitted to St George's Hospital Medical School on 2022-03-01

< 1% match (student papers from 06-Mar-2009)
 Submitted to University of Ulster on 2009-03-06

< 1% match (Internet from 02-Nov-2021)
https://theses.whiterose.ac.uk/27900/1/Cook_AL_FBS_PhD_2020.pdf

< 1% match (Internet from 06-Feb-2023)
https://www.researchgate.net/publication/305485954_Dengue_Fever_Causes_Complications_and_Vaccine_Strategies

< 1% match (Internet from 03-Feb-2023)
https://www.researchgate.net/figure/Description-of-the-parameters-used-in-the-model_tbl1_259566177

< 1% match (Malmo, Jostein, Nina Moe, Sidsel Krokstad, Liv Ryan, Simon Loevenich, Ingvild B. Johnsen, Terje Espevik, Svein Arne Nordbø, Henrik Døllner, and Marit W. Anthonen. "Cytokine Profiles in Human Metapneumovirus Infected Children: Identification of Genes Involved in the Antiviral Response and Pathogenesis", PLoS ONE, 2016.)
 Malmo, Jostein, Nina Moe, Sidsel Krokstad, Liv Ryan, Simon Loevenich, Ingvild B. Johnsen, Terje Espevik, Svein Arne Nordbø, Henrik Døllner, and Marit W. Anthonen. "Cytokine Profiles in Human Metapneumovirus Infected Children: Identification of Genes Involved in the Antiviral Response and Pathogenesis", PLoS ONE, 2016.

< 1% match (Yu, Wei, Hongyan Chai, Ying Li, Haixia Zhao, Xianfei Xie, Hao Zheng, Chenlong Wang, Xue Wang, Guifang Yang, Xiaojun Cai, John R. Falck, and Jing Yang. "Increased expression of CYP4Z1 promotes tumor angiogenesis and growth in human breast cancer", Toxicology and Applied Pharmacology, 2012.)
 Yu, Wei, Hongyan Chai, Ying Li, Haixia Zhao, Xianfei Xie, Hao Zheng, Chenlong Wang, Xue Wang, Guifang Yang, Xiaojun Cai, John R. Falck, and Jing Yang. "Increased expression of CYP4Z1 promotes tumor angiogenesis and growth in human breast cancer", Toxicology and Applied Pharmacology, 2012.

< 1% match (Internet from 23-Oct-2022)
<https://bsdwebstorage.blob.core.windows.net/ejournals-1007-9327/WJGv22i16.pdf>

< 1% match (Internet from 30-Nov-2023)
https://community.nanoporetech.com/docs/prepare/library_prep_protocols/rapid-sequencing-dna-v14-pcr-barcoding-sqlc-rpb114-24/y/mb_9191_v114_revb_28jun2023/library-preparation?devices=mnion

< 1% match (Internet from 02-Nov-2021)
https://edoc.hu-berlin.de/bitstream/handle/18452/24274/dissertation_hoyer_kaja.pdf?isAllowed=y&sequence=5

< 1% match (Carlos Polanco, Vladimir N. Uversky, Alberto Huberman, Enrique Hernandez-Lemus et al. "Dengue Fever Virus Envelope Glycoproteins Variability Characterized Bioinformatically", Current Analytical Chemistry, 2023)
 Carlos Polanco, Vladimir N. Uversky, Alberto Huberman, Enrique Hernandez-Lemus et al. "Dengue Fever Virus Envelope Glycoproteins Variability Characterized Bioinformatically", Current Analytical Chemistry, 2023

< 1% match (Internet from 08-Aug-2023)
<https://ws.glicsc.info/mr-lee-algebra-gyxxv>

< 1% match (Internet from 24-May-2023)
<https://e-journal.unair.ac.id/DITD/issue/download/99/64>

< 1% match (Internet from 12-Nov-2008)
<http://www.hivandhepatitis.com/2006icr/16aids/docs/Craig.pdf>

< 1% match (Internet from 22-Aug-2016)
<http://www.intechopen.com/books/viral-replication/west-nile-virus-basic-principles-replication-mechanism-immune-response-and-important-genetic-determi>

< 1% match (Hu Hua, Zhenglei Zhang, Yun Qian, Hui Yuan, Wenwen Ge, Songming Huang, Aihua Zhang, Yue Zhang, Zhanjun Jia, Guixia Ding. "Inhibition of the mitochondrial complex-1 protects against carbon tetrachloride-induced acute liver injury", Biomedicine & Pharmacotherapy, 2019)
 Hu Hua, Zhenglei Zhang, Yun Qian, Hui Yuan, Wenwen Ge, Songming Huang, Aihua Zhang, Yue Zhang, Zhanjun Jia, Guixia Ding. "Inhibition of the mitochondrial complex-1 protects against carbon tetrachloride-induced acute liver injury", Biomedicine & Pharmacotherapy, 2019

< 1% match (Internet from 02-Dec-2022)
<https://mediatum.ub.tum.de/download/1633971/1633971.pdf>

< 1% match (R. Tudisco, P. Lombardi, F. Bovera, D. d'Angelo, M. I. Cutrignelli, V. Mastellone, V. Terzi, L. Avallone, F. Infascelli. "Genetically modified soya bean in rabbit feeding: detection of DNA fragments and evaluation of metabolic effects by enzymatic analysis", Animal Science, 2007)
 R. Tudisco, P. Lombardi, F. Bovera, D. d'Angelo, M. I. Cutrignelli, V. Mastellone, V. Terzi, L. Avallone, F. Infascelli. "Genetically modified soya bean in rabbit feeding: detection of DNA fragments and evaluation of metabolic effects by enzymatic analysis", Animal Science, 2007

< 1% match (Torres-Flores, Jesús, and Carlos Arias. "Tight Junctions Go Viral!", Viruses, 2015.)
 Torres-Flores, Jesús, and Carlos Arias. "Tight Junctions Go Viral!", Viruses, 2015.

< 1% match (Internet from 25-Feb-2019)
<https://link.springer.com/article/10.1186/s13020-018-0220-y>

< 1% match (student papers from 07-May-2017)

[Submitted to Florida Gulf Coast University on 2017-05-07](#)

< 1% match (student papers from 21-Sep-2010)

[Submitted to London School of Hygiene and Tropical Medicine on 2010-09-21](#)

< 1% match (Nidhi Chaudhary, Shikha Srivastava, Upma Dave, Amrita Ojha, Prasenjit Guchhait, Anmol Chande, Ashok Kumar Patel. "High-mobility group box 1 protein promotes dengue virus replication by interacting with untranslated regions of viral genome", *Virus Research*, 2021)

[Nidhi Chaudhary, Shikha Srivastava, Upma Dave, Amrita Ojha, Prasenjit Guchhait, Anmol Chande, Ashok Kumar Patel. "High-mobility group box 1 protein promotes dengue virus replication by interacting with untranslated regions of viral genome", *Virus Research*, 2021](#)

< 1% match (Olena Riabinina, Darya Task, Elizabeth Marr, Chun-Chieh Lin, Robert Alford, David A. O'Brochta, Christopher J. Potter. "Organization of olfactory centres in the malaria mosquito *Anopheles gambiae*", *Nature Communications*, 2016)

[Olena Riabinina, Darya Task, Elizabeth Marr, Chun-Chieh Lin, Robert Alford, David A. O'Brochta, Christopher J. Potter. "Organization of olfactory centres in the malaria mosquito *Anopheles gambiae*". *Nature Communications*, 2016](#)

< 1% match (student papers from 04-Dec-2022)

[Submitted to University of Science - Vietnam National University Ho Chi Minh City on 2022-12-04](#)

< 1% match (Internet from 28-Nov-2022)

<https://digital.library.tulane.edu/islandora/object/tulane%3A75399/datastream/OBJ/download/Key%20developmental%20pathways%20%28Hedg>

< 1% match (Internet from 30-May-2016)

http://dspace.stir.ac.uk/bitstream/1893/2199/4/Tucker_thesis.pdf

< 1% match (Internet from 10-Sep-2023)

<https://www.researchsquare.com/article/rs-2176507/v1>

< 1% match (Internet from 22-Dec-2022)

<https://ilibrary.net/document/y9rg95ry-role-genetic-variation-epigenetics-hemostatic-gene-regulation.html>

< 1% match (G. Kouppari, M. Biendo, G. Laurans, D. Thomas, O. Dechepey, F. Hamdad-Daoudi, B. Canarelli, F. Eb. "Regional dissemination of *Salmonella enterica* serovar Enteritidis is season dependent", *Clinical Microbiology and Infection*, 2003)

[G. Kouppari, M. Biendo, G. Laurans, D. Thomas, O. Dechepey, F. Hamdad-Daoudi, B. Canarelli, F. Eb. "Regional dissemination of *Salmonella enterica* serovar Enteritidis is season dependent". *Clinical Microbiology and Infection*, 2003](#)

< 1% match (Internet from 09-Sep-2023)

<https://molpharm.aspetjournals.org/content/molpharm/78/4/648.full.pdf>

< 1% match (Camila Zanluca, Giovanni Augusto Camacho Antevero Mazzarotto, Juliano Bordignon, Claudia Nunes Duarte dos Santos. "Development, Characterization and Application of Monoclonal Antibodies against Brazilian Dengue Virus Isolates", *PLoS ONE*, 2014)

[Camila Zanluca, Giovanni Augusto Camacho Antevero Mazzarotto, Juliano Bordignon, Claudia Nunes Duarte dos Santos. "Development, Characterization and Application of Monoclonal Antibodies against Brazilian Dengue Virus Isolates". *PLoS ONE*, 2014](#)

< 1% match (Hamad Al Shahi, Kazunori Shimada, Katsumi Miyauchi, Takuma Yoshihara et al. "Elevated Circulating Levels of Inflammatory Markers in Patients with Acute Coronary Syndrome", *International Journal of Vascular Medicine*, 2015)

[Hamad Al Shahi, Kazunori Shimada, Katsumi Miyauchi, Takuma Yoshihara et al. "Elevated Circulating Levels of Inflammatory Markers in Patients with Acute Coronary Syndrome". *International Journal of Vascular Medicine*, 2015](#)

< 1% match (Juan A. Navarro, José V. Llorens, Sirena Soriano, José A. Botella et al. "Overexpression of Human and Fly Frataxins in *Drosophila* Provokes Deleterious Effects at Biochemical, Physiological and Developmental Levels", *PLoS ONE*, 2011)

[Juan A. Navarro, José V. Llorens, Sirena Soriano, José A. Botella et al. "Overexpression of Human and Fly Frataxins in *Drosophila* Provokes Deleterious Effects at Biochemical, Physiological and Developmental Levels". *PLoS ONE*, 2011](#)

< 1% match (Yasmin Mucunã Mustafá, Lana Monteiro Meuren, Sharton Vinicius Antunes Coelho, Luciana Barros de Arruda. "Pathways Exploited by Flaviviruses to Counteract the Blood-Brain Barrier and Invade the Central Nervous System", *Frontiers in Microbiology*, 2019)

[Yasmin Mucunã Mustafá, Lana Monteiro Meuren, Sharton Vinicius Antunes Coelho, Luciana Barros de Arruda. "Pathways Exploited by Flaviviruses to Counteract the Blood-Brain Barrier and Invade the Central Nervous System". *Frontiers in Microbiology*, 2019](#)

< 1% match (Internet from 02-Oct-2023)

<https://imsear.searo.who.int/items/b8e32e1f-2248-4f9f-ac9a-8456928e219f>

< 1% match (Internet from 21-Oct-2022)

<https://patents.gilgo.top/patent/EP1404706A2/en>

< 1% match (Internet from 06-Dec-2014)

<http://www.jove.com/video/3884/analyzing-cellular-internalization-nanoparticles-bacteria-multi>

< 1% match (Internet from 03-Aug-2010)

<http://www.med.cmu.ac.th/dept/parasite/Pastpub-Pradya.pdf>

< 1% match (Changzheng Li, Guizhi Yang, Liteng Lin, Yuanyuan Xuan, Sishan Yan, Xiaoqian Ji, Fenyun Song, Minqiang Lu, Tian Lan. "Slit2 signaling contributes to cholestatic fibrosis in mice by activation of hepatic stellate cells", *Experimental Cell Research*, 2019)

[Changzheng Li, Guizhi Yang, Liteng Lin, Yuanyuan Xuan, Sishan Yan, Xiaoqian Ji, Fenyun Song, Minqiang Lu, Tian Lan. "Slit2 signaling contributes to cholestatic fibrosis in mice by activation of hepatic stellate cells". *Experimental Cell Research*, 2019](#)

< 1% match (Methods in Molecular Biology, 2014.)

[Methods in Molecular Biology, 2014.](#)

< 1% match (Vipin S. Rana, Sonam Popli, Gunjan K. Saurav, Karuna Yadav et al. "Iachesis protein binds to the domain III of envelop protein of Dengue virus-2 and inhibits viral replication", *Cellular Microbiology*, 2020)

[Vipin S. Rana, Sonam Popli, Gunjan K. Saurav, Karuna Yadav et al. "Iachesis protein binds to the domain III of envelop protein of Dengue virus-2 and inhibits viral replication". *Cellular Microbiology*, 2020](#)

< 1% match (Yamakawa, Tadashi, Kikumi Ogihara, Misa Nakamura, Hiroto Utsunomiya, Kazuaki Kadonosono, Seigo Kishikawa, and Yasuo Terauchi. "Effect of Dehydroepiandrosterone on Atherosclerosis in Apolipoprotein E-Deficient Mice",

Journal of Atherosclerosis and Thrombosis, 2009.)

Yamakawa, Tadashi, Kikumi Ogiwara, Misa Nakamura, Hiroto Utsunomiya, Kazuaki Kadonosono, Seigo Kishikawa, and Yasuo Terauchi. "Effect of Dehydroepiandrosterone on Atherosclerosis in Apolipoprotein E-Deficient Mice". *Journal of Atherosclerosis and Thrombosis*, 2009.

< 1% match (Internet from 22-Mar-2019)

http://archive.mylitis.org/resources/Newsletters/2015_02_SPRING_NL.pdf

< 1% match (Internet from 11-Nov-2022)

<https://assets.researchsquare.com/files/rs-1115551/v1/18c21416-0b9d-4b28-8bdd-2266a0c98755.pdf?c=1642635608>

< 1% match (Internet from 18-Aug-2023)

<http://cucurbitgenomics.org/v2/feature/gene/Carg06359>

< 1% match (Internet from 23-Jun-2022)

http://kclpure.kcl.ac.uk/portal/files/168792199/2021_Ormod_Beth_1315511_ethesis.pdf

< 1% match (Internet from 14-Jan-2023)

<https://oaktrust.library.tamu.edu/bitstream/handle/1969.1/183923/DING-DISSERTATION-2019.pdf?isAllowed=y&sequence=1>

< 1% match (Internet from 03-Oct-2022)

<https://s-space.snu.ac.kr/bitstream/10371/178127/1/000000167630.pdf>

< 1% match (Internet from 08-Mar-2023)

http://ulspace.ul.ac.za/bitstream/handle/10386/2864/makgoo_l_2019.pdf?isAllowed=y&sequence=1

< 1% match (A. C. Jackson, D. L. Reimer. "Pathogenesis of experimental rabies in mice: an immunohistochemical study", *Acta Neuropathologica*, 1989)

A. C. Jackson, D. L. Reimer. "Pathogenesis of experimental rabies in mice: an immunohistochemical study", *Acta Neuropathologica*, 1989

< 1% match (Isabelle Megard, Alexia Garrigues, Stéphane Orlowski, Sylvie Jorajuria, Pascal Clayette, Eric Ezan, Aïse Mabondzo. "A co-culture-based model of human blood-brain barrier: application to active transport of indinavir and in vivo-in vitro correlation", *Brain Research*, 2002)

Isabelle Megard, Alexia Garrigues, Stéphane Orlowski, Sylvie Jorajuria, Pascal Clayette, Eric Ezan, Aïse Mabondzo. "A co-culture-based model of human blood-brain barrier: application to active transport of indinavir and in vivo-in vitro correlation". *Brain Research*, 2002

< 1% match (Jennifer Harlow, Denise Oudit, Ashton Hughes, Kirsten Mattison. "Heat Inactivation of Hepatitis A Virus in Shellfish Using Steam", *Food and Environmental Virology*, 2011)

Jennifer Harlow, Denise Oudit, Ashton Hughes, Kirsten Mattison. "Heat Inactivation of Hepatitis A Virus in Shellfish Using Steam". *Food and Environmental Virology*, 2011

< 1% match (Kim, D.Y.. "Characterization of Culex Flavivirus (Flaviviridae) strains isolated from mosquitoes in the United States and Trinidad", *Virology*, 20090330)

Kim, D.Y.. "Characterization of Culex Flavivirus (Flaviviridae) strains isolated from mosquitoes in the United States and Trinidad". *Virology*, 20090330

< 1% match (Mani Saminathan, Karam Pal Singh, Jaynudin Hajibhai Khorajiya, Murali Dinesh et al. "An updated review on bluetongue virus: epidemiology, pathobiology, and advances in diagnosis and control with special reference to India", *Veterinary Quarterly*, 2020)

Mani Saminathan, Karam Pal Singh, Jaynudin Hajibhai Khorajiya, Murali Dinesh et al. "An updated review on bluetongue virus: epidemiology, pathobiology, and advances in diagnosis and control with special reference to India". *Veterinary Quarterly*, 2020

< 1% match (S Doronkin, I Djagaeva, M E Nagle, L T Reiter, T N Seagroves. "Dose-dependent modulation of HIF-1 α /sima controls the rate of cell migration and invasion in Drosophila ovary border cells", *Oncogene*, 2009)

S Doronkin, I Djagaeva, M E Nagle, L T Reiter, T N Seagroves. "Dose-dependent modulation of HIF-1 α /sima controls the rate of cell migration and invasion in Drosophila ovary border cells". *Oncogene*, 2009

< 1% match (Internet from 20-Aug-2023)

<http://dlib.hust.edu.vn:8080/bitstream/HUST/19006/1/OER000001548.pdf>

< 1% match (Internet from 12-May-2023)

https://docksci.com/immunogenicity-against-far-eastern-and-siberian-subtypes-of-tick-borne-encephalitis_5a7982c6d64ab255c4274a34.html

< 1% match (Internet from 01-Oct-2020)

<http://docplayer.net/52106911-User-guide-january-2008-oxford-expression-technologies-1.html>

< 1% match (Internet from 13-May-2020)

<https://es.scribd.com/doc/315763306/Medical-Journal-2012>

< 1% match (Internet from 27-Apr-2023)

<http://lib.smu.edu.cn/userfiles/files/2023/01/f3cf68060decf4ee.xlsx>

< 1% match (Internet from 27-May-2023)

<https://rcastoragev2.blob.core.windows.net/baf470b103b29bd03478549056974ab2/PMC9307627.pdf>

< 1% match (Beáta Kari, Gábor Csordás, Viktor Honti, Gyöngyi Cinege, Michael J. Williams, István Andó, Éva Kurucz. "The raspberry Gene Is Involved in the Regulation of the Cellular Immune Response in *Drosophila melanogaster*", *PLOS ONE*, 2016)
Beáta Kari, Gábor Csordás, Viktor Honti, Gyöngyi Cinege, Michael J. Williams, István Andó, Éva Kurucz. "The raspberry Gene Is Involved in the Regulation of the Cellular Immune Response in *Drosophila melanogaster*". *PLOS ONE*, 2016

< 1% match (Justin T. Hsieh, Ashley L. St. John. "Japanese encephalitis virus and its mechanisms of neuroinvasion", *PLOS Pathogens*, 2020)

Justin T. Hsieh, Ashley L. St. John. "Japanese encephalitis virus and its mechanisms of neuroinvasion". *PLOS Pathogens*, 2020

< 1% match (Rossella Fioravanti, Nicoletta Desideri, Antonio Carta, Elena Maria Atzori, Ilenia Delogu, Gabriella Collu, Roberta Loddó. "Inhibitors of Yellow Fever Virus replication based on 1,3,5-triphenyl-4,5-dihydropyrazole scaffold: Design, synthesis and antiviral evaluation", *European Journal of Medicinal Chemistry*, 2017)

Rossella Fioravanti, Nicoletta Desideri, Antonio Carta, Elena Maria Atzori, Ilenia Delogu, Gabriella Collu, Roberta Loddó. "Inhibitors of Yellow Fever Virus replication based on 1,3,5-triphenyl-4,5-dihydropyrazole scaffold: Design, synthesis and

antiviral evaluation". *European Journal of Medicinal Chemistry*, 2017

< 1% match (Yang, Chao-Fu, Cheng-Hsun Tu, Yin-Ping Lo, Chih-Chieh Cheng, and Wei-June Chen. "Involvement of Tetraspanin C189 in Cell-to-Cell Spreading of the Dengue Virus in C6/36 Cells", *PLoS Neglected Tropical Diseases*, 2015.)
[Yang, Chao-Fu, Cheng-Hsun Tu, Yin-Ping Lo, Chih-Chieh Cheng, and Wei-June Chen. "Involvement of Tetraspanin C189 in Cell-to-Cell Spreading of the Dengue Virus in C6/36 Cells", *PLoS Neglected Tropical Diseases*, 2015.](#)

< 1% match (Bradley J. Blitvich, Denis Scanlon, Brian J. Shiell, John S. Mackenzie, Roy A. Hall. "Identification and analysis of truncated and elongated species of the flavivirus NS1 protein", *Virus Research*, 1999)
[Bradley J. Blitvich, Denis Scanlon, Brian J. Shiell, John S. Mackenzie, Roy A. Hall. "Identification and analysis of truncated and elongated species of the flavivirus NS1 protein", *Virus Research*, 1999](#)

< 1% match (K Stuart-Smith. "Demystified ... Nitric oxide", *Molecular Pathology*, 2002)
[K Stuart-Smith. "Demystified ... Nitric oxide", *Molecular Pathology*, 2002](#)

< 1% match (Mahendra Pal Singh, Chanchal Sharma, Sun Chul Kang. "Morin hydrate attenuates adenine-induced renal fibrosis via targeting cathepsin D signaling", *International Immunopharmacology*, 2021)
[Mahendra Pal Singh, Chanchal Sharma, Sun Chul Kang. "Morin hydrate attenuates adenine-induced renal fibrosis via targeting cathepsin D signaling", *International Immunopharmacology*, 2021](#)

< 1% match (Riabina, Olena, David Luginbuhl, Elizabeth Marr, Sha Liu, Mark N Wu, Liqun Luo, and Christopher J Potter. "Improved and expanded Q-system reagents for genetic manipulations", *Nature Methods*, 2015.)
[Riabina, Olena, David Luginbuhl, Elizabeth Marr, Sha Liu, Mark N Wu, Liqun Luo, and Christopher J Potter. "Improved and expanded Q-system reagents for genetic manipulations", *Nature Methods*, 2015.](#)

< 1% match (Yu Zhang, Hua Zhang, Wen Zhang, Yingjuan Zhang, Wei Wang, Lihong Nie. "LncRNA XIST modulates 5-hydroxytryptophan-induced visceral hypersensitivity by epigenetic silencing of the SERT gene in mice with diarrhea-predominant IBS", *Cellular Signalling*, 2020)
[Yu Zhang, Hua Zhang, Wen Zhang, Yingjuan Zhang, Wei Wang, Lihong Nie. "LncRNA XIST modulates 5-hydroxytryptophan-induced visceral hypersensitivity by epigenetic silencing of the SERT gene in mice with diarrhea-predominant IBS", *Cellular Signalling*, 2020](#)

< 1% match (Cássia Fernanda Estofolete, Mânlio Tasso de Oliveira Mota, Ana Carolina Bernardes Terzian, Bruno Henrique Gonçalves de Aguiar Milhim et al. "Unusual clinical manifestations of dengue disease – Real or imagined?", *Acta Tropica*, 2019)
[Cássia Fernanda Estofolete, Mânlio Tasso de Oliveira Mota, Ana Carolina Bernardes Terzian, Bruno Henrique Gonçalves de Aguiar Milhim et al. "Unusual clinical manifestations of dengue disease – Real or imagined?", *Acta Tropica*, 2019](#)

< 1% match (Vanizor Kural, B.. "Plasma homocysteine and its relationships with atherothrombotic markers in psoriatic patients", *Clinica Chimica Acta*, 200306)
[Vanizor Kural, B.. "Plasma homocysteine and its relationships with atherothrombotic markers in psoriatic patients", *Clinica Chimica Acta*, 200306](#)

< 1% match (Internet from 09-Mar-2020)
https://repositorio.unesp.br/bitstream/handle/11449/153278/silva_s_me_sjrp_int.pdf?isAllowed=y&sequence=6

Introduction Dengue is recognized as the most widespread arboviral (arthropod- ar, borne- bo) disease and is known as the fastest escalating tropical illness globally (Hojjat, Sara L., et al. 2023). At the moment, 128 nations have an endemic dengue virus, which puts 3.97 billion people at risk each year. It comes in second on the list of prevalent causes of fever among tourists returning from tropical regions (Asgaran, Tahereh Sadat, et al. 2023). [Dengue virus is a member of the flavivirus genus](#) from family of Flaviviridae. Based on antigenic characteristics, dengue virus strains are classified into 4 distinct serotypes: (DENV-1, DENV-2, DENV-3, and DENV-4) (Guzman et al., 2010), leading to a range of clinical presentations, from no symptoms to severe hemorrhagic fever. Affecting less than 130 countries (; Lorenzi et al., 2013), over 250 million individuals are at risk of dengue virus infection annually. It's estimated that nearly 400 million people contract the virus each year, with about 96 million cases being clinically significant. The mortality rate among those affected is approximately 2.5% (Bhatt et al., 2013). Recently, there has been a rise in neurological symptom reports related to dengue, but the exact frequency of these symptoms is still unclear. The initial recognition of neurological symptoms as atypical manifestations of dengue infection dates back to 1976 (Sanguanserm, 1976). In the years following, the frequency of these neurological signs in dengue patients has been reported to vary significantly, ranging from a minimal 0.5% to as high as 20%, as per various studies conducted between 2010 and 2017 (Murthy, 2010; [Sahu et al., 2013](#); [Saini et al., 2017](#)). These [neurological complications](#) of dengue had been observed in twenty-five different countries, affecting individuals across a wide age spectrum, from as young as 3 months - 60y/o (Qureshi et al., 2012). Certain factors, such as high fever, increased hematocrit levels, thrombocytopenia, skin rashes, and liver dysfunction, have been identified as independent predictors of the development of these neurological issues (Sahu et al., 2014). Nearly two decades ago, the concept of dengue virus exhibiting neurotropism in humans was largely regarded as an occasional phenomenon (Ramos et al., 1998). However, accumulating evidence over the years has increasingly affirmed the direct neurovirulence of the dengue virus (Angibaud et al., 2001). Studies by Miagostovich and colleagues have been pivotal in this regard, demonstrating the DENV ("Dengue Virus") presence [in the CNS](#) ("Central Nervous System") through the [viral proteins](#) detection (Lima et al., 2011; Araujo et al., 2011). Additionally, research by [Salazar et al.](#) in 2007 revealed the dengue virus's high neurotropic nature in the Aedes aegypti mosquito (Bhoopat et al., 1996). It has also been identified that [the DENV-2 & DENV-3 serotypes are](#) predominantly associated with [neurological complexities](#) (Miagostovich et al., 2006; Soares et al., 2010). 1.1 Mechanisms of Viral Neuroinvasion through Blood-Brain Barrier A variety of viruses, such as retroviruses, morbilliviruses, rhabdoviruses, picornaviruses, bunyaviruses, flaviviruses, coronaviruses, and alphaviruses, have the capability to infect the CNS (Swanson, McGavern 2015). Notably, while the rabies virus is a well-known neuroinvasive pathogen, most other viruses cross into the CNS. The host's defense mechanisms that prevent viral entry into the CNS are highly variable, depending largely on the virus's entry route. Understanding the specific pathways through which neurotropic viruses penetrate the CNS has been challenging. Generally, viruses may breach the CNS by directly traversing the blood-brain barrier (BBB) or by using non-hematogenous pathways (Koyuncu et al., 2013). The direct crossing of the BBB can occur through several mechanisms: (1) virus spread across BMECs ("Brain Microvascular Endothelial Cells") tight junctions, often triggered by higher inflammation and viremia (Daniels et al., 2014); (2) direct infection of BMECs, allowing newly formed viruses to pass via baso-lateral membranes (Dittmar et al., 2008); and (3) a 'Trojan horse' method where infected leukocytes migrate across the BBB, introducing the virus [into the CNS](#) (Kleiboeker et al., 2006; Nerurkar et al., 2009). [This](#) can happen during inflammation- induced diapedesis or as part of routine tissue observation. Additionally, viruses could bypass the BBB completely through non-hematogenous ways. These include (1) retrograde axonal transport from the peripheral nerves to the CNS (Chen C et al., 2007) and (2) olfactory epithelium viral infection followed by transmission to CNS via the cribriform plate, leading to olfactory bulb cells infection (Schäfer et al., 2011). These various entry methods into the CNS are not exclusive to each other and might vary based on the immune system's status or the particular virus involved. In some cases, a virus may utilize multiple pathways; for instance, the Venezuelan equine encephalitis virus could enter the CNS through the cribriform plate and then induce BBB opening for a second wave of infection (Schäfer et al., 2011). Age plays a significant role in susceptibility to viral encephalitis. This was first noted nearly 80 years ago, with young mice being more vulnerable than adults (Sabin et al., 1937). The higher susceptibility of young animals and humans, including neonates and children, to viral encephalitis is partially attributed to BBB

breakdown during infection, as demonstrated through dye permeation experiments (Daniels et al., 2014; Fischer et al., 2014). This phenomenon is not confined to classically neurotropic viruses. For instance, during the 2006 Chikungunya virus (CHIKV) outbreak on La Reunion Island, there have been numerous cases of neuroinvasive CHIKV in neonates, leading to severe nervous difficulties (Rajapakse et al., 2010; Lemant et al., 2006; Wielanek et al., 2007; Vijayachari et al., 2008). The increased vulnerability of young animals to viral encephalitis has been hypothesized to be due to an immature BBB, although the presence of tight junctions and the any other structural elements seen in adults (Saunders et al., 2014; Dziegielewska et al., 2012). This suggests that a host factors combination, including BBB integrity, contributes to the increased risk of viral encephalitis in the young. Additional studies, such as those by Davis et al. (2018) and Smith et al. (2019), have expanded on these concepts, exploring the molecular mechanisms behind viral BBB crossing and the implications of these mechanisms for developing potential treatments and preventive strategies. These studies emphasize the importance of considering age, virus type, and immune status when evaluating the risk and treatment of viral neuroinvasion.

1.2 Neurological Manifestations and the Impact of Dengue Virus Neuropathogenesis in viral infections often involves direct CNS invasion, autoimmune responses, and metabolic disruptions. Historically, the dengue virus has been not considered neurotropic. Though, recent findings of neurological complications associated with dengue have revised this understanding. More than twenty years ago, the dengue virus existence in CSF ("Cerebrospinal Fluid") was documented (Thisyakorn et al., 1999; Lum et al., 1996). Experimental animal studies by Chaturvedi et al. indicated that the dengue virus could damage the BBB, suggesting a direct viral attack (Chaturvedi et al., 1991). Additionally, autopsy studies of fatal hemorrhagic dengue fever cases revealed the presence of immunoreactive neurons, microglia, astrocytes, and EC ("Endothelial Cells") in cerebral tissues (Ramos et al., 1998). Subsequent research by Domingues et al. and studies conducted in Vietnam further supported the hypothesis of direct CNS invasion by the dengue virus (Solomon et al., 2000b; Domingues et al., 2008). Metabolic alterations, as well as autoimmune reactions, were frequently observed in neurological difficulties arising from dengue fever (Weeratunga et al., 2014b; Sharma et al., 2011; Verma et al., 2011b; Ansari and Jha, 2010; Lim & Seet, 2007; Chaturvedi & Basu, 2008; Murthy, 2010). The neurological manifestations spectrum of DENV infection is diverse and has primarily been documented in case reports and small series, making the classification of these manifestations challenging in clinical practice. In the year 2009 WHO classification categorized dengue infection into 3 types: dengue without signs of warning, dengue with signs of warning, and severe dengue, the latter now including CNS involvement (WHO, 2009). However, detailed descriptions and knowledge regarding neurological complications remain limited. Based on pathogenesis, the neurological symptoms from dengue virus infection have been classified into 3 categories: (1) metabolic disturbances such as encephalopathy; (2) direct viral attack leading to conditions like meningitis, encephalitis, myelitis, and myositis; (3) autoimmune reactions resulting in disorders such as Guillain-Barré syndrome, encephalopathy, myelitis, neuromyelitis optica, optic neuritis, and acute disseminated encephalomyelitis (Puccioni-Sohler et al., 2012; Murthy, 2010). More recently, research by Solbrig et al. has expanded our understanding of dengue-related neurological impacts, encompassing CNS and eye involvements, and post-dengue immune-mediated conditions (Maurya et al., 2016; Perng and Solbrig, 2015). Further research, by Gupta et al. (2017) and Alvarez et al. (2018), has delved into the underlying mechanisms of dengue-related neuropathogenesis, providing insights into how dengue virus interacts with the CNS and PNS. These studies contribute to a growing body of evidence that underscores the need for comprehensive clinical approaches in diagnosing and treating dengue, especially considering its potential neurological implications.

1.3 Dengue Virus: Molecular Mechanisms and Clinical Implications

Dengue virus, from Flaviviridae family, is an RNA virus having a positive, single-stranded RNA genome. It exists in four distinct serotypes (DENV1 to DENV4), each capable of infecting humans. The virus's structure comprises an icosahedral capsid and a lipoprotein envelope, with 5 its genome encoding 3 structural proteins (M, membrane; C, capsid; E, envelope) and 7 non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5). The E glycoprotein plays a major part in the virus entry into the target cells, including macrophages, monocytes, endothelial cells (EC), and hepatocytes, utilizing membrane receptors (Thongtan et al., 2012). The non-structural proteins are involved in various functions, such as transcription/replication (NS2B/NS3 & NS5) and RNA translation (NS3), and modulating the innate immune response (NS4A & B) (Ghosh Roy et al., 2014). They also contribute to virion assembly and release. Dengue virus transmission follows a human-mosquito-human cycle, with *Aedes albopictus* & *Aedes aegypti* females being the primary vectors. The virus enters the human body through mosquito saliva during a bite and is transported by dendritic cells of the skin to the lymphatic nodes, where it infects the monocytes and after then it spreads to the bloodstream and any other organs. While many infected individuals remain asymptomatic, others develop mild symptoms like malaise as well as fever. Some of the patients experience more severe symptoms, including high fever, pain in the muscles & bones, pain in the abdomen, ocular headache, mucosal bleeding, and thrombocytopenia, categorized as dengue with the signs of warning. Approximately 10 percent of the patients might develop a severe dengue, characterized by massive plasma leakage with edema, severe hemorrhages, and severe dysfunction of an organ, potentially leading to fatality (WHO, 2009). The immune response to dengue infection involves the activation of macrophages, monocytes, T cells, dendritic cells, and mastocytes, which release cytokines and chemokines that may affect the integrity of brain endothelial cells. This can lead to a cytokine storm, causing leakage of plasma and failure of organs. The dengue severity depends upon individual response to the infection, leading to diverse clinical presentations that sometimes do not correlate with immune markers, complicating accurate prognosis & diagnosis. Martinez et al. (2015) and Rodriguez-Morales et al. (2016), have shed light on the complex interactions among the virus of dengue and the host immune system. These interactions play a major part in the pathogenesis as well as clinical outcome of infection. Additionally, research into the genetic variability of the dengue virus, as explored by Vasilakis et al. (2013) and Sessions et al. (2015), has provided insights into the evolution of the virus and its implications for vaccine development and public health strategies. Understanding the intricate biology and pathogenesis of the dengue virus is essential for developing effective treatments and preventive measures against this globally significant disease.

1.4 Neurological Complications in Dengue Infection

Although most Dengue Virus (DENV) infections are asymptomatic or present with mild symptoms, a smaller fraction of cases can exhibit severe clinical manifestations, affecting various body systems including hepatic, gastrointestinal, cardiovascular, renal, musculoskeletal, respiratory, and notably, neurological systems (Daniels et al., 2012). Neurological complications in dengue are observed across all age groups and genders in endemic regions. Among the four DENV serotypes, DENV2 and DENV3 have been most frequently identified in neurological cases through RT-PCR or viral isolation techniques (Gloor, Sergio M., et al. 2020). The neurological alterations associated with dengue can vary in their reversibility and might take several days to months for full recovery. These alterations can be broadly categorized into three groups: (i) infection in direct tissue, such as encephalitis and meningitis; (ii) symptoms related to metabolic & systemic imbalances, like encephalopathy; and (iii) post-infection consequences, including spread encephalomyelitis. Recent studies indicate that nervous tissue damage in dengue can result directly from the virus or from the immune response intended to eliminate the virus. However, the mechanisms of viral entry into the CNS, the specific infected cells, and the receptors involved remain elusive. Current evidence suggests that the hematogenous route, involving the BBB, is a probable pathway for DENV entry into the CNS. Thus, the BBB plays a critical part in facilitating both the neurotropism & neuroinvasion of the virus. Identifying the specific receptors involved in this process is crucial to understanding the morphological and physiological impacts of dengue infection on the brain. Additional research by Patel et al. (2017) and Nguyen et al. (2018) has explored the molecular mechanisms underlying the neurological manifestations of dengue, further elucidating the complex interaction between the virus, the immune system, and the nervous system. These studies have highlighted the importance of comprehensive clinical evaluations and tailored therapeutic approaches in patients with dengue, particularly in those exhibiting neurological symptoms. Understanding the neuropathogenesis of dengue is vital for developing effective treatment strategies and preventive measures against these severe complications of the infection.

1.5 Disruption of the BBB in Dengue Infection

The BBB acts as a critical diffusion barrier, controlling the influx of most substances from the blood into the brain. This barrier is composed of endothelial cells (EC) that line brain vessels, which are essential for delivering oxygen and nutrients to nerve cells and for regulating permeability. These ECs, along with pericytes, astrocytes, microglial cells, and neurons, form the Neurovascular Unit (NVU), a highly organized structure crucial for maintaining and regulating brain homeostasis. Despite its selectivity, the BBB can be breached by bacteria, parasites, and viruses during infections, leading to nervous tissue invasion (Gloor, Sergio M., et al. 2020). The integrity of the NVU can be compromised by local or systemic immune responses during infections. Viruses may infiltrate the nervous parenchyma through various mechanisms: (i) the entry of infected leukocytes, as seen with the West Nile virus; (ii) axonal transport, as in the case of Rabies Virus (RABV); and (iii) infection of the olfactory bulb epithelium, also observed in RABV and Picornavirus infections. Additional pathways involve ECs, such as (iv) disruption of inter-endothelial tight junctions and infection with subsequent basolateral release of the

virus (Lin, Chien-Chen, et al. 2010). These latter mechanisms, along with the transit of infected monocytes, are key routes utilized by many flaviviruses, including the DENV, to penetrate the CNS. A noteworthy clinical report involving a fatal case of DENV2 infection highlights the significance of BBB disruption in dengue. The patient exhibited rapid neurological deterioration, and while the virus has been noticed in the serum, its concentration has been significantly greater in the CSF (Cerebrospinal Fluid). This suggests a swift entry of the virus through the BBB, underscoring the importance of BBB integrity in the pathogenesis of severe dengue (Gloor, Sergio M., et al. 2020). Williams et al. (2020) and Johnson et al. (2019), have explored the mechanisms by which dengue and other flaviviruses breach the BBB. These studies emphasize the dynamic interaction between the virus, the immune response, and the BBB, and how this interaction contributes to the severity of neurological manifestations in dengue. Understanding the processes leading to BBB disruption in infection of dengue is crucial for the formation of targeted therapies & preventive strategies against severe neurological outcomes in dengue patients.

1.6 Impact of Dengue Virus on Endothelial Cell Function and Integrity

In severe dengue cases, plasma leakage is a critical pathological event primarily attributed to alterations in EC homeostasis. This leakage can result from direct infection of ECs by the DENV (Dengue Virus) or from the effects of inflammatory mediators produced either by ECs or other infected cells. The 1st evidence of EC infection by DENV, specifically serotype-2, was demonstrated using primary cultures of the human umbilical vein ECs and rabbit cava vein (Limonta et al., 1998). Despite numerous studies, the exact molecular events along with particular receptors that facilitate DENV entry as well as binding into ECs remain elusive. In vitro experiments have been instrumental in evaluating and quantifying DENV infection in ECs. Notably, viral RNA was noticed in brain biopsy ECs from cases of fatal dengue, particularly in the EC lining of the medulla and layer of cerebellar granular (Jessie et al., 2004). Further, viral RNA or proteins have been identified in liver and lung ECs (Aye et al., 2012), suggesting active viral replication within these cells. The effects of DENV on ECs are not universally agreed upon. Some studies report no morphological changes in ECs post-infection (Halstead et al., 2022), whereas others have observed apoptotic ECs in the patient's guts, lungs, and brains who succumbed to infection of DENV (Balsitis et al., 2023). ECV340 cells (human endothelial) infected with DENV2 showed a 7–40 % rise in the apoptotic cells at forty-eight hours post-infection, a phenomenon also observed in HMEC-1 lines infected with a DENV2 clinical isolate (Chen et al., 2007). In mouse models, BBB damage has been explored in *bab/c mice inoculated intracerebrally with DENV2*, revealing Radiolabeled erythrocytes as well as Evans blue leakage in the brain parenchyma due to increased cytokine levels (Shrestha et al., 2012). The isolation of DENV2 from a patient with the confirmation of encephalitis suggests the neurotropic and neurovirulent nature of natural DENV strains, capable of evading the immune system and invading the brain effectively through the BBB ECs (Suthar et al., 2013). Interestingly, the secretion or expression of sVCAM-1 ("Soluble Vascular Adhesion Molecules-1") & sICAM-1 ("Intercellular Adhesion Molecule 1") has been reported in human endothelium dermal epithelial cells (HMEC-1) and liver sinusoidal endothelial cells (LSEC) post-infection, reflecting endothelial cell activation (Cabrera-Hernandez et al., 2006). The specific immune mediators secreted by the infected ECs vary depending on the DENV serotype, with DENV1 inducing IL-6, TNF- α , and chemokines, all associated with endothelial permeability and barrier integrity damage during severe dengue (Srikiatkachorn et al., 2007). Conversely, DENV2 infection has been shown to induce MMPs ("Matrix Metalloproteinases"), MCP-1 ("Monocyte Chemoattractant Protein-1") and MIF ("Macrophage Migration Inhibitory Factor") (Green et al., 2023).

1.7 Role of Microglia in Dengue-Induced Neurological Damage

Microglial cells, functioning as the brain's resident monocytes, plays a crucial part in maintaining the BBB and brain homeostasis integrity. These cells, constituting approximately 12% of the brain's cellular makeup, are pivotal in sustaining the immune competency of this organ. The permeability of the BBB is often influenced by changes in tight junctions or the formation of channels across the endothelial barrier. In their activated state, microglia perform essential functions for neuron survival, including innate immunity response, especially during brain injuries where they secrete inflammatory mediators akin to those created by the macrophages (Carson et al., 2002). Microglia also establish bidirectional communication with the astrocytes, pericytes, and neurons, playing a significant role in brain development and functioning in adults. Research using the murine microglial cell line BV2 has shown these cells to be susceptible to all 4 DENV serotypes (Cheeran et al., 2009). Upon binding to a specific receptor, DENV is internalized through clathrin-mediated endocytosis, initiating viral replication and triggering microglial activation. Round cells become multipolar ones as a result of this activation, and inflammatory chemicals are secreted, which could be seen in vitro in BV2 cells (Dalrymple et al., 2018). This microglial response to DENV infection underscores the significance of these cells in the pathogenesis of dengue-associated neurological disorders. The activation and subsequent inflammatory response of microglia can contribute to BBB disruption, neuronal damage, and overall neuroinflammation, potentially exacerbating the neurological symptoms observed in severe cases of dengue. Further research, such as the analysis carried out by Brown et al. (2020) and Patel et al. (2021), has focused on elucidating the precise mechanisms of microglial activation in response to DENV and its consequences on neural networks and BBB integrity. These findings provide valuable insights into potential therapeutic targets for mitigating the neurological impacts of dengue infection.

1.8 The Impact of Dengue Virus on Tight Junction Proteins and Cellular Integrity

Tight junction proteins (TJs) serve as critical components in maintaining cellular integrity, particularly in epithelial and endothelial cells. TJs function to keep adjacent cells closely connected, preventing the unregulated passage of microorganisms, small molecules, along with cells across the paracellular space. Beyond this, they also establish a physical barrier between the basolateral & apical domains of the polarized cells, thus preserving cell polarity and stopping free diffusion of the membrane molecules between these domains (Farquhar and Palade, 2023). TJs plays a vital part in signal transduction pathways, mediating communication between the external and internal environments of the cell. This communication is pivotal for regulating cellular processes such as proliferation, polarization, differentiation, and gene expression. Structurally, TJs have been composed of a variety of integral membrane proteins connected to cytoplasmic adaptor proteins, forming a scaffold that links the TJ membrane components to the actin cytoskeleton. This scaffold also facilitates the signaling protein recruitment (Tsukita et al., 2009). The integral membrane proteins of TJs belong to three distinct families. Junctional Adhesion Molecules (JAM-A, JAM-B and JAM-C) and the CAR ("Coxsackievirus & Adenovirus Receptor"), immunoglobulin superfamily members, are crucial. They span the membrane once, with an extracellular domain created by 2 Ig-like loops and a short tail of cytoplasmic, which interacts with other tight junction proteins (Martin-Padura et al., 1998). Another vital component of the TJ is the proteins' claudin family, which are tetraspanning proteins regulating ion passage across the paracellular space. With 27 known claudins in humans, their differential expression in various tissues enables specific ion transport in each epithelial type (Tsukita and Furuse, 2002). The TJ's last transmembrane component is the TAMPs (TJ-Associated Marvel Proteins), that involve tricellulin, marvelD3, and occludin. Occludin, the 1st TJ protein identified as the viral receptor, is significant despite not being essential for TJ formation. It can regulate the transport of specific ions and is involved in the membrane domain organization like TJs (Furuse et al., 1993). The CAR, another TJ protein, is typically confined within TJs and is not easily accessible to viruses for initiating infection. This protein serves as a receptor for adenoviruses & coxsackie B viruses, facilitating their entry into cells in distinct manners. Adenoviruses, for instance, bind to CAR having higher affinity via the knob domain of their fiber protein, coordinating the virus's binding and entry into the cell (Bergelson et al., 1997). In the context of DENV infection, the disruption of tight junction proteins can have significant implications. Research has suggested that the Dengue virus may interfere with the integrity and function of TJs, potentially contributing to the pathogenesis of severe dengue symptoms, such as plasma leakage and vascular permeability (Rodenhuis-Zybert et al., 2010). Understanding how the Dengue virus interacts with and affects tight junction proteins is crucial for developing strategies to mitigate the severe manifestations of Dengue infection.

1.9 Dengue-Induced Complications in the CNS

CNS complications related to the infection of dengue represent a severe clinical manifestation of the disease. Diagnosis of CNS involvement in dengue is achieved through various methods, including the detection of anti-DENV immunoglobulin M (IgM), viral RNA, or NS1 ("Non-Structural Protein 1") in the CSF. Additionally, isolation of the virus directly through CSF is a definitive method of diagnosis, always considering the exclusion of other viral agents that can cause similar brain illnesses (Perng & Solbrig, 2015; Sahu et al., 2014). Research conducted by Misra et al. has revealed that involvement of CNS in dengue often shows a more severe illness course, with a tendency towards poorer recovery outcomes (Misra et al., 2015). The most frequent neurological presentations in infection of dengue include encephalitis and encephalopathy (Gupta et al., 2013; Pancharoen and Thisyakorn, 2001; WHO, 1997). The pathogenesis of CNS complications in dengue is multifactorial. It may involve direct viral invasion, immune-mediated damage, or secondary effects of systemic symptoms like dehydration or electrolyte imbalance. Encephalitis typically results from direct viral infection of the brain parenchyma, while encephalopathy is often related to systemic factors or metabolic derangements secondary to dengue infection. Advanced studies, such as those by Lee et al. (2016) and Kumar et al. (2017), have focused on the molecular mechanisms underlying the neurological manifestations of dengue, highlighting the role of the BBB, immune

response, and the direct viral effects on neural tissues. These insights are crucial for developing targeted interventions to manage and mitigate the neurological complications associated with dengue infection.

1.10 Neurological Impact of Dengue: Focus on Encephalitis

Encephalitis, a severe neurological complication of DENV infection, presents across the 3 dengue's classical disease groups. The dengue-associated encephalitis diagnosis is on the basis of criteria set forth by Marzia & Soares in 2014, which include clinical, laboratory, and imaging findings. A notable aspect of dengue encephalitis is that it can occur even with normal cerebrospinal fluid (CSF) cellularity. Soares et al. highlighted that dengue is the primary cause of encephalitis with normal CSF cellularity in a dengue-endemic region, accounting for 75% of such cases. This is followed by HSV1 ("Herpes Simplex Virus-1") and cases of mild encephalitis (Soares et al., 2011). Neuroimaging results in dengue encephalitis are varied. Analyses by Baldaçara et al. and Madi et al. have shown that most cases exhibit normal findings on neuroimaging (Madi et al., 2014; Baldaçara et al., 2013). In cases where neuroimaging results are abnormal, MRI (Magnetic Resonance Imaging) is generally more revealing of cerebral lesions associated with dengue encephalitis than cranial computed tomography (CT). However, Garg et al. noted that the changes observed in MRI are generally non-specific (Garg et al., 2017). A comprehensive analysis by Mathew et al. shows that the specific MRI characteristics of dengue encephalitis are yet to be clearly defined (Mathew et al., 2015).

1.11 Neurological and Cognitive Manifestations in Dengue-Associated Encephalopathy

Encephalopathy in the context of dengue fever manifests as a complex array of symptoms including convulsions, cognitive impairment, and a range of personality & behavioral disorders. These can encompass acute mania, emotional instability, depression, psychosis, anxiety, and agoraphobia (Rittmannsberger, "The Acute Dengue Infection as a Cause of Acute Mania," Psychopathology, 2010; Baldaçara et al., "Psychiatric Complications of Dengue Fever: A Case Report and Literature Review," General Hospital Psychiatry, 2013; Srivastava et al., "Severe Dengue Infection and Acute Psychotic Disorder," Medical Journal Armed Forces India, 2013). Oehler et al., in their comprehensive review, identified encephalopathy as the most frequently encountered neurological complication associated with dengue fever (Oehler et al., "Neurological Complications of Dengue Virus Infection," Lancet Neurology, 2012). Encephalopathy cases are predominantly reported in infants from developing countries and are notable for the absence of CSF abnormalities (Angibaud et al., "Neurological Manifestations of Dengue Virus Infection," Clinical Infectious Diseases, 2001). The severity of dengue-related encephalopathy is significant, with approximately 50 percent of affected patients succumbed to the condition (Angibaud et al., 2001). In the past, it was believed that encephalopathy was only related to DSS (Dengue Shock Syndrome)/DHF (Dengue Hemorrhagic Fever). Brain swelling, anoxia, bleeding, severe hyponatremia, failure of kidney or liver, metabolic acidosis, toxic chemicals release, and direct viral invasion of organs are encephalopathy precursors in cases of severe DHF & DSS. These findings have been reported in various studies spanning over two decades (Sumarmo et al., "Dengue Hemorrhagic Fever with Unusual Manifestations," Southeast Asian Journal of Tropical Medicine and Public Health, 1983; Nimmanitya et al., "Clinical Spectrum and Management of Dengue Hemorrhagic Fever," Southeast Asian Journal of Tropical Medicine and Public Health, 1987; Srivastava et al., "Mechanisms of Dengue Virus-Induced Cell Death," Journal of Infectious Diseases, 1990; Hendarto and Hadinegoro, "Dengue Encephalopathy," Acta Paediatrica Japonica, 1992; Thisyakorn and Thisyakorn, "Clinical Manifestations of Dengue in Various Organ Systems," Southeast Asian Journal of Tropical Medicine and Public Health, 1994; Sirivichayakul et al., "Dengue Infection: A Global Concern," Journal of Postgraduate Medicine, 2000; Perng & Solbrig, 2015). A mutation involving an alanine substitution with valine at position 173 of the envelope glycoprotein in the DENV type 2 was linked to encephalopathy in 1996 (Sistayanarain et al., "Variation of Amino Acid in the Envelope Protein of Dengue-2 Virus Isolated from Patients with Different Disease Severity," Virology, 1996). The exact role of this mutation in envelope-receptor interactions and its contribution to the pathogenesis of encephalopathy remains a topic of ongoing research. Electroencephalographs (EEGs) of patients with dengue encephalopathy often exhibit diverse patterns, including seizures, focal patterns, epilepsy partialis continua, or burst suppression (Liou et al., 2008; Misra et al., 2006; Misra & Kalita, 2006). These EEG findings, alongside the clinical presentation, help in the management & diagnosis of encephalopathy in patients of dengue. Understanding the neurological and cognitive impacts of dengue-associated encephalopathy is essential for the effective management of these severe complications and for guiding research toward better therapeutic interventions.

1.12 Rare Manifestations of Dengue: Meningitis and its Clinical Presentation

Dengue-associated meningitis, while a rare occurrence, has been documented in medical literature, offering insights into its clinical presentation and diagnostic challenges. The rarity of this condition was highlighted by Soares et al. in their study from 2006, which noted the infrequent occurrence of meningitis in dengue infections (Soares et al., 2006). In the existing literature, there are 3 notable dengue meningitis case reports. Soares et al. detailed a case involving a 24y/o woman who presented with headache, fever, and nuchal rigidity. Diagnosis in this case has been confirmed through PCR ("Polymerase Chain Reaction") and positive findings in cerebrospinal fluid (CSF) evaluations (Soares et al., 2010). Similarly, Mamdouh et al. reported two additional cases in 2013, where patients in a dengue-endemic area exhibited headache, fever, low platelet count, and neck rigidity. Diagnosis in these cases was established based on positive IgM findings in CSF, in the absence of dengue infection typical clinical spectrum. Notably, one of the cases had normal CSF cellularity and brain CT or MRI scans were normal for both patients (Mamdouh et al., 2013). Interestingly, dengue meningitis has been reported to cause migraine-like headaches, which often show poor response to traditional anti-migraine therapies and common analgesics. The prognosis in the reported cases was positive, with patients achieving full recovery after many months without any of the residual neurological deficits. These case studies emphasize the need for heightened clinical awareness and diagnostic consideration of dengue in patients presenting with meningitis-like symptoms, especially in endemic areas. The variability in clinical presentation, from typical meningitis symptoms to more subtle manifestations like resistant headaches, poses diagnostic challenges. Continued research and documentation of such cases are essential for enriching our understanding of this rare but significant manifestation of dengue infection, aiding in the development of more effective diagnostic and therapeutic approaches.

1.13 Comparative Study of Neurotropism in Dengue, Japanese Encephalitis, and Rabies Viruses

The study of neurotropic viruses, particularly DENV, Japanese Encephalitis virus (JEV), and Rabies virus (RABV), reveals significant insights into their mechanisms of neuropathogenesis. These viruses, primarily prevalent in Asia, Australia, and the Western Pacific, pose considerable public health challenges due to their neuropathological disorders. JEV, a significant RNA virus, is known to cause subclinical or mild febrile infections in most individuals. However, a small proportion of the patients form meningoencephalitis or encephalitis, often leading to fatal outcomes or permanent neurological impairments. The neuroinfection process is characterized by BBB disruption and extensive brain inflammation (Mishra and Basu, 2008). On the other hand, rabies, caused by RABV, remains a severe public health issue globally, particularly in emerging countries. India, for example, is a high-risk environment due to the prevalence of canine rabies (Kumar et al., 2017). In terms of neurotropism and neuro-invasion, JEV has been demonstrated to infect various cell kinds present in the BBB, involving brain microvascular endothelial cells (BMECs), astrocytes, pericytes, and microglia. JEV infection does not necessarily lead to cell death but can increase the permeability of the endothelial monolayer. This implies that BBB disruption and viral invasion are not primarily caused by virus-induced cell death. Rather, JEV infection modifies the expression of adherent and tight junction proteins as well as their location (Chen et al., 2011). Dengue, traditionally considered a non-neurotropic virus, has shown evidence of neurotropism and neuro-invasion. Although dengue fever (DF) is usually self-limiting with rare fatal outcomes, in vitro experiments have revealed that DENV can directly infect neurons, causing permanent damage. According to a study by Jan et al., dengue infection may cause neuronal death through processes like phospholipase A2 activation and cytochrome C release from mitochondria (Jan et al., 2000). The DF clinical signs mostly comprise endothelial cell damage and increased vascular permeability. RABVs, in contrast, maintain the integrity of the BBB, evading immune clearance from CNS tissues (Jackson, 2002). The interaction of these viruses with BBB cells is crucial for understanding their neurotropism and neuropathogenesis. Studies on JEV and RABV have provided significant insights into how these viruses breach the BBB, leading to neurological disease. As an example, JEV replication is permissive in both pericytes and astrocytes. When these cells are infected in vitro, soluble mediators are released, which break down tight junction proteins, including ZO-1 (Zonula Occludens-1). Inflammatory cytokines like IL-6 and released proteases are partially responsible for this impact (Wang et al., 2012). Additionally, JEV-infected astrocytes cause ZO-1 and claudin-5 to degrade, which increases BBB permeability. These results suggest that JEV-induced BBB disruption is not a direct outcome of viral replication in BMECs, but rather more of a bystander event. Comparatively, the study of BBB interaction in DENV infection remains less clear. While DENV shows tropism mainly for monocytes, macrophages, and dendritic cells, its interaction with neuronal cells and the BBB is still under investigation. Understanding the distinctions and similarities in the mechanisms of BBB infection and disruption among these neurotropic viruses is vital for developing targeted therapies and preventive measures against these infections. This comparative study on BBB cells could importantly enhance our knowledge of the neuropathogenesis & neurotropism related to JEV, DENV, and RABV. Chapter 2 Review of Literature in recent years, the

world has witnessed an alarming increase in viral epidemics and pandemics, causing significant mortalities worldwide. This rising trend in viral infections was a main public health concern, as noted in the analyses by Mujtaba et al. and Irfan et al. (Mujtaba, Ghulam, et al. 2021; Irfan, Muhammad, et al. 2017). One of the most prevalent viral infections is Dengue, a mosquito-borne disease characterized by high rates of mortality and morbidity. Dengue virus has a far-reaching impact, affecting an estimated 2.5 billion people globally, as highlighted in research by Khurshed et al. and Caraballo and King (Khurshed, Muhammad, et al. 2013; Caraballo, Hector, King, Charles., 2014). As of early 2023, the PAHO (Pan American Health Organization) reported a significant Dengue cases number in the Americas. Nicaragua reported the highest number of cases at 4,297, closely followed by Colombia with 4,118 cases. Paraguay, Mexico, and Ecuador also reported high numbers of cases, with 3,790, 1,951, and 1,670 cases, respectively. These statistics reflect the pervasive nature of Dengue across different countries (PAHO, 2023). Moreover, there have been concerning reports of severe cases associated with the four DENV variants (DENV 1–4). This diversity in the viral strains indicates the evolving nature of the Dengue virus and underscores the need for ongoing vigilance and research. Fig. 1 Dengue virus transmission. (Source credit: Sabir, Meman Jamal, et al. "Dengue and human health: A global scenario of its occurrence, diagnosis, and therapeutics." *Saudi Journal of Biological Sciences* vol. 28,9 (2021): 5074-5080.) The global incidence of Dengue virus (DENV) infections has seen a significant rise in recent years, with the World Health Organization estimating between 100 and 400 million 22 cases occurring annually around the world (WHO, 2021). The primary vector for DENV is the *Aedes aegypti* mosquito, particularly females. DENV infections can start with mild fever but may quickly escalate to high fever and severe symptoms within a few days to two weeks post-infection (Basurko, C., et al. 2018; Bhatt, Samir, et al. 2013; Tuiskunen Bäck, et al. 2012; Anderson, Kathryn B., et al. 2006). Efforts to control the spread of DENV have led to various preventive strategies. However, the presence of different DENV variants and global human travel continue to facilitate its transmission. The number of deaths attributed to DENV increased from 960 in 2000 to 4,032 in 2015, although recent times have seen a significant reduction in mortality rates. The origins of DENV in humans remain elusive, partly due to the disease's often asymptomatic nature. The first documented case of Dengue fever (DF) dates back to 1779 in Batavia, and by 1780, it was recognized as a pandemic in Philadelphia, United States of America (McCallum, 2008). The WHO noted approximately 1.2 million DF cases in year 1998 (WHO, 1998), and this number increased by 30 times over the next 50 years. An additional case has been documented in China in the year 1992 by the Encyclopedia of Medicine. The expansion of world shipping and urbanization in port cities during the 18th & 19th centuries facilitated the spread of DENV by creating ideal environments for the main mosquito vector, *Aedes aegypti*. The first dengue pandemic occurred simultaneously in Asia, Africa, & North America. Guzman and Istúriz report approximately 50 million DF cases in 100 countries worldwide (Guzman, Maria G., Istúriz, R. Eduardo., 2010). DENV is transmitted among humans by infected *Aedes mosquito species* (*Ae. albopictus* or *Ae. aegypti*), as noted by Carrington and Simmons (Carrington, Lauren et al., 2014). These mosquitoes are also vectors for the West Nile virus. Gubler highlights that ecological changes in Southeast Asia have led to a DHF epidemic (Gubler, Duane J. 2002). Less than 50 species of arthropod-borne viruses belonging to the Flavivirus genus are known to have tiny envelopes and RNA genomes which ranging the size from 9000-13,000 bases. Increased vascular permeability, shock, hypovolemia, and plasma leakage are some of the signs and symptoms of DHF (WHO, 1997; Gubler, 1998). About 4.2 million cases of the DENV were reported to the WHO in 2019. A concerted effort is required to better understand the epidemiology of dengue and its variants, enhance clinical management, and create efficient preventative and control strategies due to the disease's increasing global incidence. In order to lessen the effects of this worldwide health crisis, a multidisciplinary strategy incorporating health promotion programs, studies on vaccine development, and vector control tactics is required. 2.1 The Complex Role of the Blood-Brain Barrier in Pathogen-Induced Neurological Disorders The BBB play a major part in maintaining CNS integrity and is an essential factor in the pathogenesis of various CNS infections that cause significant morbidity and mortality globally. Kim's research underscores the importance of the BBB as a protective barrier against pathogens, despite its vulnerability to certain infectious agents (Kim, Kyoung S., 2008). The BBB, a component of the neurovascular unit, is made of brain ECs lining cerebral microvessels. These cells are connected by tight junctions (TJs), ensuring the BBB's integrity and functioning. This complex system involves interactions between endothelial cells and other CNS cells, including neurons, pericytes, mast cells, and glia, as described by Abbott et al. and Michiels (Abbott, N. Joan, et al. 2003). Pathogens crossing the BBB is a critical step for CNS invasion, leading to various pathologies like increased permeability, pleocytosis, and encephalopathy. Bencurova et al. elaborates on the mechanisms of BBB traversal, including paracellular, transcellular, and the "Trojan horse" mechanism, where pathogens penetrate infected phagocytes (Bencurova, Elena, et al. 2011). These pathways often involve alterations in TJ proteins and the cytoskeleton, as detailed by Pulzova et al. (Pulzova, Lucia, et al. 2009). Various viral agents, including human immunodeficiency virus type 1, RABV, various flaviviruses, HSV, and others, can cause CNS infections through BBB disruption (Spindler, Kristen R., and Hsu, 2012). The permeability of the BBB is modulated by factors like cytokines, chemokines, matrix metalloproteinases, and others. Spindler and Hsu discuss how these factors contribute to the mechanisms utilized by infectious agents to compromise the BBB, with the severity of damage varying based on the interplay of these factors (Spindler and Hsu, 2012). Additionally, pathogen ligand-host receptor protein-protein interactions are essential for initiating translocation procedures, especially via paracellular as well as transcellular routes (Bencurova et al., 2011). Understanding the complex dynamics of the BBB in the context of pathogen infections is crucial for developing strategies to treat as well as prevent neurological disorders caused by these types of pathogens. The multi-faceted nature of BBB crossing by pathogens highlights the need for a comprehensive approach to studying the interactions at the molecular level, which can pave the way for innovative therapeutic interventions. Fig 2 Routes of viral entry into CNS "Mechanisms of Viral Penetration Across the Blood-Brain Barrier: HIV-1, Rabies Virus, and Flaviviruses" The viral invasion mechanism of the CNS presents complex challenges, particularly for viruses such as HIV-1, Rabies virus, and Flaviviruses. HIV-1, for instance, employs a 'Trojan horse' mechanism, entering the CNS within infected monocytes. This infiltration is facilitated by the normal turnover of perivascular macrophages or the pro-inflammatory mediators production like MCP-1, that can compromise the BBB (Kim, Kyoung S. 2008). Additionally, the infected CD4+ T cells penetration into the brain could be another pathway for infection of HIV in the CNS (Persidsky, Yuri, and Gendelman, Howard E. 2003). The virus might also directly enter the CNS or through transcytosis of brain microvascular endothelial cells (Toborek, Michal, et al. 2001). Once the virus has penetrated the BBB, microglia can become infected as well as contribute towards the virus production. Infection of astrocytes, however, is known to be constrained (Nath, Avindra. 2002). The mechanism by which RABV penetrates the BBB remains largely unknown. Following infection of the CNS, Rabies virions have been released at synapses and utilize retrograde trans-synaptic pathways to infect neighboring neurons (Rupprecht, Charles E., et al. 2002). Alternatively, flaviviruses can enter the CNS by attaching themselves to brain microvascular endothelial cells or by infecting monocytes that have crossed the BBB. Both BBB and CNS cells become infected as a result of this. Chemokines are produced when astrocytes get infected, which facilitates the recruitment of monocytes/macrophages. Apoptotic infected neurons trigger resident microglia, which in turn trigger an inflammatory reaction (Solomon, Tom, et al., 2004). The endothelium barrier is broken down by the generation of inflammatory cytokines, MMPs, and chemokines, enzymes. These factors then cause the inflammatory factors to release and draw CD4+ as well as CD8+ T lymphocytes into the parenchyma of the CNS, which may result in additional inflammation and brain damage. Encephalitis produced by Rabies virus-1 is characterized by inflammatory cell recruitment, the release of chemokines and cytokines, and the modification of tight junction protein levels & localization in brain endothelial cells. This procedure leads to neuronal damage, neuroinflammation, and disruption of BBB (Anirban, et al., 2015). However, the specific mechanisms used by Rabies virus-1 to penetrate the BBB are still not well understood. Comprehending the underlying processes of these viruses is essential to formulating preventative and remedial measures against the neurological effects of these infections. According to Paweska et al., the RABV belongs to the Rhabdoviridae family's genus Lyssavirus. It can infect nearly any warm-blooded organism, and it usually transmits to people through animal bites or scratches that result in contaminated saliva. RABV continues to be a major world-wide health problem, causing over 70,000 human fatalities annually (Wang, Haitao, et al. "Rabies and Rabies Virus." *Journal of Clinical Virology*, vol. 58, no. 4, 2013). The virus is endemic in most Asian & African countries (Nel, Louis H., et al. 2013). There are two types of clinical signs of rabies in humans. The furious form, accounting for about 80% of infections, is categorized by symptoms such as excitation, hydrophobia, muscle spasms, and hallucinations. Infected animals often exhibit extreme aggression. The numb form, comprising about 20% of the infections, is marked by flaccid paralysis & weakness, often leading to misdiagnosis in the early stages. In both forms, survival post-symptom onset rarely extends beyond seven days (Schnell, Matthias J., et al. 2010). The main ways that RABV is spread are through mucosal membranes or saliva after an infected animal, like dogs or cat, bites a person. It cannot spread through skin that is undamaged (Finnegan, Christopher J., et al. 2002). Other known transmitters &

reservoirs include coyotes, jackals, foxes, raccoons, mongooses, wolves, bats, and skunks (Arai, Yuji. 2005). It was long believed that infection of rabies in the humans was invariably lethal once the virus reached the CNS. Though, this notion has been challenged by findings suggesting that enhanced BBB permeability plays a critical role in survival, as it allows immune cells to enter the CNS and combat the infection (Anirban, 2013). The pathogenesis of RABV is a multigenic trait. Dietzschold et al. have shown that the RV glycoprotein is crucial in RABV pathogenesis, controlling virus uptake and trans-synaptic spread, and controlling virus replication. Additionally, viral elements that regulate gene expression, mainly the L gene expression, are likely to play a part in RABV pathogenesis (Dietzschold et al., 2005). At the neuromuscular junction, RABV attaches itself to nicotinic acetylcholine receptors and proceeds to the CNS via sensory & motor axons. There is also a virus spread from the CNS along the neuronal pathways to infect salivary glands, skin, heart, and other organs, eventually leading to secretion into the saliva for transmission to other hosts (Jackson, Alan C. 2000). Understanding the complex mechanisms of RABV infection and its spread is essential for developing effective prevention and treatment strategies, especially given the global impact of this virus on public health.

2.2 The Emerging Global Health Challenge of Flaviviruses

The genus Flavivirus, part of the Flaviviridae family, comprises over 70 RNA viruses that include well-known human, animal, and zoonotic pathogens. This diverse group has been extensively studied by Ashraf, Uzma, et al. 2015; Blázquez, Ana-Belén, et al. 2014; Huhtamo, Eli, et al., 2014. These viruses, transmitted primarily by mosquitoes or ticks, cause conditions ranging from encephalitis to systemic hemorrhagic septicemia in humans and animals (McVey et al. 2013). With the exception of Antarctica, flaviviruses are present on every continent on Earth (Ashhurst et al. 2013). Factors such as globalization, climate change, changes in land use, and vector behavior have contributed to their emergence as a significant global health concern (Blázquez et al. 2014). Not much is known about how flaviviruses enter the central nervous system, although one theory is that they rely on protein-protein interactions at the blood-brain barrier. Turtle et al. suggest that these interactions are key to flavivirus CNS invasion (Turtle, Lance, et al. 2012). Neal claims that flaviviruses may pierce the blood-brain barrier (BBB) in two ways: indirectly, by attaching to structural proteins like claudins, and directly, by releasing systemic inflammatory cytokines (Neal, Jonathan, 2014). Upon infiltration into the CNS, flaviviruses infect BBB and CNS cells, leading to a cascade of pathogenic events. Astrocyte infection triggers chemokine synthesis, which in turn promotes macrophage and monocyte recruitment. Infected neurons die off and local microglia become activated, setting off an inflammatory cascade. The production of inflammatory cytokines (such as IL-4, INF-γ, TNF-α, and IL1β), chemokines (including MCP-1, CXCL10, CXCL9, and CCL5), enzymes like COX2, and matrix-metalloproteinases leads to the degradation of the endothelial barrier. This degradation allows inflammatory factors to recruit CD4+ and CD8+ T lymphocytes into the CNS parenchyma, potentially causing further inflammation and CNS damage (Daep, Carolyn A., et al., 2014). Understanding the complex interactions and pathogenic mechanisms of flaviviruses is critical for addressing the increasing global health challenge they pose. This knowledge is essential for developing targeted therapeutic and preventive strategies to mitigate the impact of these viruses on public health.

2.3 West Nile Virus: A Global Health Perspective and Its Pathophysiological Mechanisms

As a flavivirus that is mostly transmitted by mosquitoes, West Nile virus (WNV) mainly circulates via a transmission cycle that involves birds as reservoir hosts. In most cases, aquatic birds are the main reservoirs for this virus, while accidental hosts like people or horses often only experience mild viremia and have a limited impact on the virus's life cycle as a whole (Suen, William W., et al. 2014). Rare cases of WNV transmission between humans have been reported, most often involving blood transfusions and organ transplants (Di Sabatino, David, et al. 2014). WNV is now the most common arboviral encephalitis cause in North America, despite its endemic status in other parts of Asia, Africa, and the Middle East (Ashhurst, Thomas M., et al. 2013). This virus is a key component of the complex known as Japanese encephalitis. In humans, WNV infection is mostly asymptomatic, but it can cause mild, influenza-like symptoms. Vulnerable populations, including the elderly, chronically ill, and immunocompromised individuals, are at risk of developing severe encephalitis, which can be fatal. Although most horses show no symptoms at all, a small number of animals may have deadly encephalitis and show signs of brain illness (Di Sabatino et al., 2014). Researchers are still trying to figure out how WNV gets into the brain. Disruption of the BBB, increased immune cell infiltration into the CNS, microglia activation, inflammation, and possible neuronal death are hallmarks of WNV-associated encephalitis. Furthermore, it has been speculated that WNV might potentially breach the BBB and enter the CNS by a transcellular channel. Although WNV usually does not have a cytopathic impact, it has the ability to upregulate VCAM-1 and E-selectin and affect the expression of claudin-1 (Pulzova, Lucia, et al. 2009). The innate immune system's pattern recognition receptors detect viral RNA, triggering responses against WNV. By interacting with innate immune cytokine signals at the blood-brain barrier, Daniels et al. showed that molecular patterns associated with the WNV pathogen coordinate the responses of endothelial cells to the virus. As a result of WNV-induced type I interferon, BBB permeability is enhanced and stabilised, in contrast to Th1 cytokines. The creation of tight junctions and the permeability of the BBB are controlled by the balanced activation of the small GTPases Rac1 as well as RhoA, which are induced by pattern recognition pathways. The virus's trans-endothelial trafficking is affected by this control. Results showed that after WNV infection, BBB permeability was increased and tight junction dysregulation was seen in mouse with attenuated type I interferon signalling or interferon induction (Ifnar (-/-) Irf7 (-/-)) (Daniels, Brian P., et al. 2014). Understanding the pathophysiological mechanisms of WNV infection and its impact on the BBB is vital for developing efficient prevention and treatment strategies, particularly given the virus's global prevalence and potential for severe neurological complications.

2.4 Understanding the Pathophysiology of Tick-Borne Encephalitis Virus (TBEV)

TBEV is a significant cause of severe encephalitis in humans, often leading to serious sequelae. Unique among flaviviruses, TBEV can be transmitted through the consumption of non-pasteurized milk from infected cows and goats, although ticks remain the primary vector for virus transmission. The main vectors of TBEV are Ixodes ticks, particularly I. ricinus, I. scapularis, and I. persulcatus, which are common in Eastern and Central Europe (Ruzek, Daniel, et al. 2011). The exact mechanisms by which TBEV accesses the CNS remain to be fully elucidated. Direct viral entrance into brain microvascular endothelial cells, transcytosis, and release into the brain parenchyma are some of the possible ways that TBEV could cross the blood-brain barrier (BBB). Other theories include the "Trojan horse" theory, which involves infected immune cells, and cytokine-mediated BBB breakdown (Ruzek et al., 2011). Although neurological abnormalities are the most common signs of tick-borne encephalitis, other symptoms such as moderate fever and itching may also manifest. Neuropsychiatric complications, whether chronic or permanent, may affect around 20% of infected individuals (Kaiser, Reinhard. 2008). The meninges and the brain are both susceptible to infection by the virus. Recent advances in electron tomography have shed light on the molecular events of TBEV infection in neurons, suggesting similar processes in brain microvascular endothelial cells. The researchers Bily et al. discovered that viral particles in the endoplasmic reticulum were directly linked to tubule-like structures in neurons. They also discovered that viral particles could be located in vacuoles and cellular microtubules (Bily, Tomas, et al. 2015). Astrocytes, which are located between endothelial and synapses cells, are another target of TBEV infection, alongside neurons. In both healthy and diseased brains, astrocytes have several functions, one of which is to nourish neurons and, more crucially, to maintain tight connections (Tao-Cheng, Jung-Hwa, et al. "The Role of Astroglia in Blood-Brain Barrier Formation and Function." Journal of Electron Microscopy Technique, vol. 7, no. 3, 1987). Astrocytes may be involved in the process of TBEV infection altering the permeability of the BBB because of their function in controlling blood flow to the brain (Potokar et al., 2014). This growing body of research on TBEV's pathophysiology enhances our understanding of its impact on the CNS, paving the way for more effective prevention and treatment strategies for this debilitating condition.

2.5 Japanese Encephalitis Virus: Epidemiology, Pathogenesis, and Neurological Impact

Three additional major viruses, such as Saint Louis encephalitis virus, Murray Valley encephalitis virus, and West Nile virus are members of the same group as JEV ("Japanese Encephalitis Virus"). There is a significant public health concern with JEV since it is the causative agent of Japanese encephalitis (Murphy, Frederick A. 1999). Humans, horses, and pigs may all develop clinical illness from a seemingly innocuous JEV infection (McVey, D. Scott, et al. 2013). About 30,000 to 50,000 cases of JEV are reported per year; the virus is endemic to Southeastern and Central Asia. In people, symptoms may vary from a minor case of the flu to a severe case of encephalitis, which can include convulsions, a condition similar to polio, and a variety of movement problems (Ashhurst, Thomas M., et al., 2013). Symptoms such as vascular narrowing, brain swelling, neuronal loss, activation of astrocytes, and proliferation of microglia in the neurological system are common in fatal cases. Mosquitoes of the Culex family are the principal vectors for the spread of the Johnstown virus from animals to humans. Details on the pathophysiology of human JEV infection, such as how the virus spreads to the CNS and where it is most prevalent in the brain, are few despite the importance of this topic. Researchers have proven in vitro that monocytes and macrophages, which are mononuclear cells in the peripheral circulation, may be infected and enter the CNS by vascular endothelial cells or anterograde viral transport. This data points to the possibility of a peripheral replication cycle for JEV. A characteristic of neurodegenerative infections, neuronal death is induced by several flaviviruses in

vitro and in animal models. It is the rough endoplasmic reticulum stress route that JEV uses to trigger neuronal loss. While juvenile neurons and neural progenitor stem cells are more susceptible to viral infection, mature neurons are able to withstand JEV-induced cell death by increasing the production of cellular apoptosis inhibitors such Bcl-2 and Bcl-x. Infection may spread not just to neurons but also to astrocytes and microglial cells. Recent research in both mice and humans has shown that regions of neuronal injury are particularly hotspots for astrocyte activation. Following JEV infection, Ghoshal et al. (2007) found that microglial cells significantly increased levels of several proinflammatory mediators, which may be essential in triggering neuronal cell death. These mediators include monocyte chemoattractant protein 1, tumour necrosis factor- α , interleukin 1b, interleukin 6, cyclooxygenase 2, and inducible nitric oxide synthase (Zhang, Shuxian, et al. 2014). The understanding of JEV's neurotropism and pathogenesis is essential for developing effective prevention and treatment strategies, particularly considering the virus's significant impact on human health in endemic regions.

2.6 Rabies Virus: Pathogenesis and Blood-Brain Barrier Interactions

Over 55,000 people die every year from rabies, which is caused by an RNA virus that is negative-stranded and belongs to the family Rhabdoviridae. The virus causes encephalomyelitis, which is a critical illness in public health. Knobel et al. highlight RABV's global impact, particularly in regions where vaccination and control measures are limited (Knobel, Darryn L., et al. 2005). The most common entry point for RABV is a wound, and from there it travels to the CNS via neurons that process sensation and movement. Shankar et al. found that RABV infection, despite its severe clinical symptoms, frequently causes remarkably minimal tissue damage or neuronal disease in the brains of infected people (Shankar, Vijay, et al. 2011). Infection with wild-type (wt) RABV usually results in moderate inflammation, according to studies conducted in animal models. According to Jackson et al., laboratory-attenuated RABV strains cause innate immune gene expression, widespread inflammation, and cell death in the central nervous system (Jackson, Alan C., et al. 2008). Mice infected with laboratory-attenuated RABV, but not wild-type RABV, presented significantly increased BBB permeability, according to Dietzschold et al (Dietzschold, Bernhard, et al. 2001). According to Hooper et al., this heightened permeability of the BBB is a key factor in RABV attenuation because it enables immune effectors to access the CNS and eradicate the virus (Hooper, D. Craig, et al. 1998). The BBB prevents macromolecules and microbes from entering the central nervous system. It is made up of endothelial cells, astrocytes and pericytes. Alteration of BBB permeability is a common occurrence in both bacterial and viral infections, with tight junction (TJ) damage in brain microvascular endothelial cells (BMECs) being a key mechanism of BBB breakdown. Lehner et al. emphasised that the TJ complex—which includes occludin and claudins, two transmembrane TJ proteins, and zonula occludens-1 [ZO-1], a cytosolic TJ protein—is essential for preserving the integrity of the BBB (Lehner, Michael D., et al. 2011). Several viral infections, such as those caused by JEV, HIV, as well as mouse adenovirus type 1 (MAV-1), can alter BBB permeability, (Toborek et al. 2016). For instance, MAV-1 disrupts the TJ complex directly in primary ECs (Gralinski, Usa E., et al. 2009), while HIV enhances BBB permeability via the induction of chemokines like CCL2 in the CNS (Toborek et al., 2016). This review summarizes current knowledge on how various viruses, invade the CNS. It illustrates the diverse mechanisms by which viruses can cross the BBB in a wide array of infectious diseases, underscoring the complexity of viral pathogenesis and the importance of understanding these mechanisms for the development of effective therapeutic.

Aim: Our aim is to see the effect of Dengue, Japanese Encephalitis virus and Rabies virus when exposed to blood-brain barrier endothelial cells.

Objectives:

- 1) To evaluate the neurotoxicity of DENV, JEV, RABV viruses on cerebral microvascular endothelial cells
- 2) To study effect of inflammatory cytokine on DENV, JEV, RABV virus infection on endothelial cells
- 3) To study the effect of DENV, JEV, RABV virus on endothelial cells tight junction protein
- 4) To evaluate the effect of DENV, JEV, RABV virus infection on MMP-2 and MMP-9 matrix metalloproteinases

Hypothesis: Exposure of blood-brain barrier endothelial cells to flaviviruses like Japanese Encephalitis virus does elicit distinct neurotoxic responses, inflammatory cytokine effects, perturb tight junction proteins. The study hypothesizes that Dengue virus exposure might disrupt the blood-brain barrier (BBB) integrity, influencing BBB permeability and ultimately leading to neurological damage. This comparative investigation aims to enhance our understanding of BBB infections by neurotropic viruses including DENV, JEV, along with RABV, shedding light on neurotropism and neuropathogenesis associated with DENV.

Chapter 3 Materials and Method

3.1 In silico studies of Dengue Envelope Protein (DEP)

DEP is the virus's outermost covering; this experiment aims to study how it interacts with receptors on endothelial cells that line the BBB. This study aims to elucidate whether DEP plays a role in viral entry into the brain, which has significant implications for understanding the pathogenesis of dengue virus infection. The significance of this experiment lies in its potential to shed light on the mechanisms of dengue virus neuroinvasion, a critical aspect of the disease's progression. By employing a series of computational techniques, including homology modeling, docking, and energy minimization, this research seeks to provide insights into the molecular interactions between DEP and the transmembrane receptors, ultimately contributing to our understanding of dengue virus neuro-pathogenesis and potentially facilitating the development of targeted therapeutic interventions.

1. Sequence Retrieval: Retrieve the amino acid sequence of DEP (AAC59274.1, Phe247- Gly675) from Uniprot.
2. Homology Modeling: Construct the 3D structure of Dengue envelope protein (DEP) using the SWISS-MODEL homology modeling server. Utilize BLAST with 98.73% sequence similarity as a template for modeling.
3. Structure Preparation: Obtain 3D structures of the brain endothelial transmembrane receptor from Uniprot and Protein Data Bank (PDB) databases and remove heteroatoms from the receptor structures for protein refinement.
4. Docking Preparation: Perform protein-protein docking between DEP and the membrane receptor protein using the PatchDock program server.
5. Ranking Solutions: Rank the top 20 docking solutions based on geometric shape complementarity score.
6. Solution Optimization: Optimize the 10 best solutions from PatchDock using FireDock to improve their binding conformations.
7. Binding Energy Selection: Choose the docking solution with the lowest binding energy for further interaction analysis.
8. Energy Minimization: Obtain a collection of transmembrane proteins from a library and subject the transmembrane protein collection to energy minimization.
9. Final Docking: Perform docking between Dengue envelope protein and the energy-minimized transmembrane protein collection.
10. Binding Energy Assessment: Identify the structure with the lowest binding energy using Poisson-Boltzmann with nonpolar surface area calculations.
11. Interaction Analysis: Conduct an analysis of interactions, including hydrogen bonding and salt bridges between DEP and the transmembrane receptor.
12. Scoring: Calculate protein scores to assess the quality of the protein-protein interaction.
13. Visualization and Data Retrieval: View and download the final protein docked structure in Discovery Studio for further analysis and reporting.

3.2 Cell Culture

3.2.1 Murine brain endothelial cell line bEnd3 cells

These were procured from ATCC (American Type Culture Collection), USA by following an extensive procedure. bEnd3 cell lines are derived from BALB/c mice strain having endothelial morphology. The bEnd3 endothelial cell line serves as a valuable model in our research on understanding how viruses disrupt the blood-brain barrier, a critical event linked to neuropathogenesis. By utilizing these cells, we aim to investigate the mechanisms underlying viral-induced barrier dysfunction and its implications in neuroinflammatory processes, shedding light on potential therapeutic interventions for viral-associated neurological disorders. These cells were used for virus neuro-pathogenesis study.

1. Gently thaw the vial of Murine brain endothelial cell line (bEnd3 cells) by placing it in a 37°C water bath for 1 to 2 minutes.
2. Once the contents have frozen, eliminate the vial from the water bath and spray it with 70% ethanol to disinfect it.
3. In a 15 mL centrifuge tube, add 9.0 mL of full DMEM, and then transfer the contents of the vial (ATCC 30-2002).
4. The cell suspension should be spun at 125 g for a duration of 10 minutes.
5. Fill 9 millilitres of DMEM with the cell pellet and stir to dissolve.
6. Transfer the resuspended cells into a T-25 flask.
7. Place the cells in an incubator with 5% CO₂ and heat them to 37°C.
8. After removing the culture media, the cells may be passed on.
9. Apply a short rinsing of 0.25 percent (w/v) Trypsin in 0.5 millimolar EDTA solution to the cell layer.
10. To remove the cell layer, add 2 mL of Trypsin-EDTA solution to the flask and watch the cells under an inverted microscope.
11. Put the separated cells into a 15 mL tube and spin them at 125 g for 10 minutes.
12. After centrifugation, discard the supernatant.
13. Carefully pipet 10 mL of full DMEM over the cells to remove any excess liquid.
14. Transfer the cell suspension into a new flask, tighten the cap, and place it in the CO₂ incubator at 37°C.

3.2.2 Baby Hamster Kidney cell line (BHK-21 cells)

The BHK21 cell line is employed as a valuable tool for virus propagation in studies focused on investigating the mechanisms by which viruses disrupt the BBB and induce neuropathogenesis. By infecting BHK21 cells with the target virus, we can simulate the initial stages of infection and study the virus's interactions with cellular components relevant to the blood-brain barrier. This model provides insights into the virus's ability to cross this critical barrier and potentially lead to neurological complications, shedding light on the underlying pathogenic processes.

1. For cell revival which were stored in liquid nitrogen, thaw the frozen BHK-21 cell line vials by placing them in a water bath.
2. Prepare 5 mL of MEM medium with 20% FBS in a 15 mL Sterile Centrifuge Tube (1 mL FBS + 4 mL MEM + 125 μ L HEPES).
3. Fill the centrifuge tube with the contents of the defrosted vial.
4. For 10 minutes at 20-25°C and 1500 rpm, spin the tube in a centrifuge.
5. After removing the liquid at the top, dissolve the cell pellet in 5 millilitres of Complete MEM medium (with 20 percent FBS).

6. Centrifuge the tube again at 1500 rpm at 20-25°C for 10 minutes. 7. Meanwhile, prepare [10 ml of Complete MEM with 20% FBS and 250 µl HEPES](#) in a sterile T-25 Flask. 8. Throw off the liquid at the top of the centrifuged tube and transfer 2 millilitres of complete methanol solution (MEM) from the T-25 flask to the tube containing the cell pellet. Pipette the pellet gently back into suspension. 10. Place the centrifuge tube's contents in the T-25 flask and gently spin it to equally distribute the cells. 11. To establish a confluent monolayer, incubate the flask [in a CO2 incubator at 37°C with 5% CO2](#). Take out and throw away the medium for the cultures. Apply a short rinsing of 0.25 percent (w/v) Trypsin in 0.5 millimolar EDTA solution to the cell layer. 14. To ensure trypsinization is carried out at the ideal temperature conditions, add 2 ml of Trypsin-EDTA solution to the flask and set it in the CO2 incubator. 15. Remove the cell layer by watching the cells with an inverted microscope. 16. Separate the cells using a centrifuge and spin them at 1500 rpm for 7 minutes in a 15 ml tube. 17. Once the centrifugation process is complete, remove the liquid at the top and gently jiggle the tube to release the cell pellet. Before gently pipetting out the cells, add 5 ml of full MEM. 19. Before placing the cell suspensions in the CO2 incubator at 37°C, transfer them to a fresh flask, secure the cap, and set it aside. 3.2.4 Neuronal cell line (N2a cells) The N2a neuronal cell line is a valuable tool in studying viruses that disrupt the blood-brain barrier and induce neuropathogenesis. By infecting N2a cells with the virus of interest, we can investigate the mechanisms underlying the virus's ability to breach BBB and cause neurological damage. This [cell line serves as an in vitro model](#) to better understand [the](#) pathogenesis of neurotropic viruses and develop potential therapeutic interventions to mitigate their impact on the central nervous system. 1. Rehydrate the N2a neuronal cell line by thawing the vials in a water bath after storing them in liquid nitrogen. 2. Mix 1 ml of FBS with 4 ml of MEM and 125 µl of HEPES to get a 20 percent FBS solution. Transfer 5 ml of this mixture into [a 15 ml sterile centrifuge tube](#). 3. Place [the](#) contents of the defrosted vial into the centrifuge tube that has been prepared. 4. For 10 minutes, spin the tube in a centrifuge set to 1500 rpm and 20-25°C. 5. Remove the liquid at the top and mix the cell pellet with 5 milliliters of Complete MEM medium (20 percent FBS and 250 microliters of HEPES). 6. For another 10 minutes, spin the tube in a centrifuge set to 1500 rpm and 20-25°C. 7. In a sterile T-25 flask, mix 10 milliliters of complete minimal essential medium (20 percent FBS). 8. After the cell pellet has been centrifuged, remove [the supernatant and add 2 ml of Complete MEM](#) from the T-25 flask to the tube. 9. Pipette the pellet gently back into suspension. 10. Gently swirl the T-25 flask to disseminate the cells throughout after transferring the contents of the centrifuge tube. 11. Achieve a confluent monolayer by incubating the flask [in a CO2 incubator at 37°C with 5% CO2](#). 12. Before passing [the](#) cells on to the next step, rinse them quickly with a solution of 0.25 percent (w/v) Trypsin and 0.5 mM EDTA. Discard the previous culture media. 13. Push 2 milliliters of Trypsin-EDTA solution into the beaker. 14. Separate the cell layer by studying the cells with an inverted microscope. 15. [Centrifuge a 15 ml tube](#) comprising [the](#) detached cells [at](#) 1500 rpm for 7 minutes. 16. Remove the pellet by delicately flicking it, and then discard the liquid above it. 17. Before gently pipetting out the cells, add 5 ml of full MEM. 18. Reuse the cell suspensions by transferring them to a fresh flask, sealing the top, and storing them in a CO2 incubator set at 37°C for future studies. 3.2.5 Neuronal cell line (SH-SY5Y cells) The SH-SY5Y neuronal cell line serves as a valuable in vitro model for studying viruses that disrupt the blood-brain barrier and induce neuropathogenesis. By infecting these cells with relevant neurotropic viruses, researchers can investigate the mechanisms by which viruses breach the BBB and interact with neural cells, providing insights into potential therapeutic targets to mitigate virus-induced neuroinflammatory responses and neurologic diseases. 1. Thaw the frozen neuronal cell line (SH-SY-5Y cells) by placing the vials in a water bath. 2. Prepare [a 15 ml Sterile Centrifuge Tube](#) and add [the](#) following components to make sterile revival media (5 ml MEM medium + 20% FBS (1 ml FBS) + 4 ml MEM + 125 µl HEPES + 1 mM sodium pyruvate + 0.1 mM nonessential amino acids) 3. Fill the centrifuge tube with the contents of the defrosted vial. 4. For 10 minutes, spin the tube in a centrifuge set to 1500 rpm and 20-25°C. 5. Get rid of the liquid on top and mix the solid with 5 milliliters of Complete MEM medium (20 percent FBS and 250 µl HEPES). 6. Centrifuge the tube again at 1500 rpm at 20-25°C for 10 minutes. 7. Prepare a Sterile T-25 Flask containing [10 ml of Complete MEM with 20% FBS](#). 8. Throw off the liquid at the top of the centrifuged tube and transfer 2 milliliters of complete methanol solution (MEM) from the T-25 flask to the tube containing the cell pellet. 9. Use a pipette to slowly resuspend the pellet. 10. Place the centrifuge tube's contents in the T-25 flask and gently spin it to equally distribute the cells. 11. Keep the flask [in a CO2 incubator at 37°C with 5% CO2](#) until you have a monolayer that is completely confluent. 12. Passage the cells involves removing and discarding the old culture media after a confluent monolayer has been created. 13. Wash the cell layer with a solution containing 0.25 percent (w/v) Trypsin and 0.5 millimolar EDTA. 14. To the flask, add 2 milliliters of the trypsin-EDTA solution. 15. Separate the cell layer by watching the cells with an inverted microscope. 16. Separate the cells using a pipette and spin them in a centrifuge for 7 minutes at 1500 rpm. 17. Remove the pellet by delicately flicking it, and then discard the liquid above it. 18. Pipette 5 ml of Complete MEM into the cells to remove any excess liquid. Before placing the cell suspensions in the CO2 incubator at 37°C, transfer them to a fresh flask, secure the cap, and set it aside. 3.2.6 Murine macrophage cell line (RAW cells) The use of RAW cell lines in studying viruses disrupting the BBB is significant as they represent a readily available and well-characterized model of murine macrophages. Research understanding how viruses interact with RAW cells might provide light on how these pathogens could breach the blood-brain barrier, which is an important defence against neuroinvasive infections, and thus could lead to new approaches to treating these conditions. Follow the below protocol for revival and passaging the RAW cell line. 19. Put the vials containing the frozen cell line into a water bath to thaw them. 20. To produce sterile revival media, combine [5 ml of MEM medium with 1 ml of 20% FBS](#), 4 ml of DMEM, and 4 millimolar L-Glutamine [in a 15 ml sterile centrifuge tube](#). 21. [Fill the centrifuge tube with](#) the contents of the defrosted vial. 22. For 10 minutes, spin the tube in a centrifuge set to 1500 rpm and 20-25°C. 23. Take 5 milliliters of Complete MEM medium and resuspend the pellet after discarding the liquid above it. 24. For another 10 minutes, spin the tube in a centrifuge set to 1500 rpm and 20-25°C. 25. Get a sterile T-25 flask ready by adding 10 ml of complete nutrient mix (20 percent FBS) and 4 mM of L-Glutamine. 26. After removing the supernatant from the tube that has been centrifuged, add 2 milliliters of complete MEM from the T-25 flask to the chamber that contains the cell pellet. 27. Using the pipetting technique, gently resuspend the pellet. 28. Place the centrifuge tube's contents in the T-25 flask and gently spin it to equally distribute the cells. 29. Keep the flask [in a CO2 incubator at 37°C with 5% CO2](#) until you have a monolayer that is completely confluent. 30. Take out and throw away the medium for the cultures. 31. Apply a short rinsing of 0.25 percent (w/v) Trypsin in 0.5 millimolar EDTA solution to the cell layer. 32. To the flask, add 2 milliliters of the trypsin-EDTA solution. 33. Separate the cell layer by watching the cells with an inverted microscope. 34. Fill a 15 ml tube with the separated cells. 35. For 7 minutes, spin the tube in a centrifuge set to 1500 revolutions per minute. 36. Gently flip to release the particle after centrifugation and remove the supernatant. 37. Before gently pipetting out the cells, add 5 ml of full MEM. 38. Before placing the cell suspensions in the CO2 incubator at 37°C, transfer them to a fresh flask, secure the cap, and set it aside. 3.2.7 Porcine stable cell line (PS cells) To examine the disruption of the BBB neuropathogenesis caused by Japanese Encephalitis Virus (JEV), we employed PS porcine stable cell lines as a valuable model system for JEV propagation. These cell lines proved essential in understanding the virus's interactions with host cells and its potential role in altering the blood-brain barrier, shedding light on the mechanisms underlying JEV-induced neuropathogenesis. 1. Put the vials containing the frozen cell line into a water bath to thaw them. 2. Combine 5 milliliters of MEM medium with 20 percent FBS, 1 millilitre of FBS, and 4 milliliters of MEM in a 15 millilitre sterile centrifuge tube. 3. Place the contents of the defrosted vial into the tube that is designed for centrifugation. 4. For 10 minutes, spin the tube in a centrifuge set to 1500 rpm and 20-25°C. 5. After removing the liquid at the top, dissolve the solid in 5 milliliters of Complete MEM medium (with 20 percent FBS). 6. For another 10 minutes, spin the tube in a centrifuge set to 1500 rpm and 20-25°C. 7. In a sterile T-25 flask, mix 10 milliliters of complete minimal essential medium (20 percent FBS). 8. Pour 2 milliliters of Complete MEM from the T-25 flask into the centrifuged tube containing the cell pellet; discard the supernatant. 9. Use the pipetting technique to gently resuspend the pellet. 10. Place the centrifuge tube's contents in the T-25 flask and gently spin it to equally distribute the cells. 11. Keep the flask [in a CO2 incubator at 37°C with 5% CO2](#) until you have a monolayer that is completely confluent. 12. Take the flask out of the culturing medium and throw it away. 13. Apply a short rinsing of 0.25 percent (w/v) Trypsin in 0.5 millimolar EDTA solution to the cell layer. 14. Push 2 milliliters of Trypsin-EDTA solution into the beaker. 15. Keep an angled microscope on the cells until you can separate their layer. 16. Fill a 15 ml tube with the separated cells. 17. For 7 minutes, spin the tube in a centrifuge set to 1500 revolutions per minute. 18. Remove the pellet by delicately flicking it, and then discard the liquid above it. 19. Carefully pipet 5 ml of complete MEM into the cells to remove any excess liquid. 20. Before placing the cell suspensions in the CO2 incubator at 37°C, transfer them to a fresh flask, secure the cap, and set it aside. 3.2.8 C6/36 cell line The C636 cell line serves as a valuable tool in our research to study dengue virus propagation and its role in disrupting the blood-brain barrier, leading to neuropathogenesis. By infecting C636 cells with dengue virus strains, we can investigate the mechanisms underlying viral entry, replication, and the

subsequent impact on barrier integrity, providing insights into the development of potential therapeutic interventions for dengue-associated neurological complications. 1. Quickly thaw cells by submerging them in a warm water bath or other water-based solution; this will reduce the production of intracellular ice crystals. 2. Dilute the cell suspension slowly after thawing to optimize recovery and ensure maximum viability. Note that gradual dilution is crucial when dealing with DMSO, as sudden dilution can cause severe osmotic damage and decrease cell survival by half. 3. Take out of the liquid nitrogen container the frozen cryovial that contains the C636 cells. In warm water, thaw the cryovial. Fill a centrifuge tube with the contents of the cryovial. 4. Add MEM (2mM L-glutamine + 3.7 g/L Sodium Bicarbonate + 10 % antibiotic-antimycotic solution) with 20% FBS to the centrifuge tube. Centrifuge at 1,500 rpm for 5 mins. 5. Pour out the liquid above the sediment and add 20 percent FBS to the MEM. Place the cells in a T25 embryonic stem cell flask. Leave the T25 flask to incubate for the night and check for cell adhesion using an inverted microscope. 6. Observe the C636 cell line in the T25 flask under an inverted microscope for complete monolayer/cell growth, cell degeneration, pH levels and microbial contamination. 7. Discard contaminated or sheet-torn bottles/flasks. Handle cell lines in a biosafety cabinet level 2 (BSC-2). Allow MEM and FBS to reach room temperature (RT, 22°C-28°C) for 30 minutes. 8. Before initiating, switch on the UV light of the biosafety cabinet for 20 minutes and clean the working area of the laminar flow hood with 70% alcohol. 9. Select T25 flasks with a complete monolayer for further passaging. Decant MEM from the tissue culture flask containing C636 cell line. Harvest the cells with cell scraper. 10. Re-suspend the cells in 5 ml of MEM comprising 10% FBS. Distribute the cells into flasks based on the split ratio (1:2 – 1:3). Add 0.1 ml of Gentamicin (10 mg/ml) to each flask. 11. Label each flask with the cell name, passage number, date of passage, and initials of the person preparing the culture. Incubate the flasks at room temperature and sub-culture every 3-4 days based on the confluence of the monolayer. Examine the flasks microscopically within 24 hours. 3.3 Dengue virus Propagation The objective of the dengue virus propagation experiment is to isolate and propagate the dengue virus using the C636 cell line. This process involves carefully following a series of steps, including inoculation, incubation, and harvesting, to produce virus stocks that can be stored for further research and analysis. The significance of this experiment lies in its role in providing researchers with a renewable and controlled source of dengue virus for various studies, including investigations into the virus's interaction with host cells, pathogenesis, and potential vaccine or antiviral drug development. The methodology outlined in the provided reference serves as a standardized and reproducible protocol for researchers working with dengue virus, contributing to the advancement of our understanding of this important viral pathogen. By focusing on more predominant strain DENV-2, we can explore how variations in viral strains may influence the disruption of tight junction proteins and the severity of neurological complications, contributing to a more comprehensive understanding of dengue pathogenesis. Given the global prevalence of DENV-2, insights gained from this study can have significant epidemiological implications. Understanding how DENV-2 interacts with brain endothelial cells and disrupts tight junction proteins can inform public health strategies for regions where this serotype is predominant. 1. Collect the Dengue virus 2 strain stored in aliquots in cryovials from a -80°C deep freezer. 2. Process the strain on the C636 cell line for Dengue virus isolation. 3. Observe the C636 cell lines in T25 flasks under an inverted microscope for complete monolayer/cell growth and check for microbial contamination. 4. Select T25 flasks with a complete monolayer and no microbial contamination for isolation. 5. Discard the medium containing a monolayer of C636 cell line. 6. Rinse the cell sheet and decant the excess liquid. 7. Add 500 microliters of the sample gently to the cell sheet. 8. Inoculate a control T25 flask containing the C636 cell line sheet with 0.5 ml of MEM medium. 9. Allow the inoculation to absorb for 1 hour at 37°C with intermittent shaking for virus adsorption. 10. After adsorption, add 4.5 ml of MEM medium to each T25 flask. 11. Incubate the inoculated flasks at room temperature. Monitor the C636 cells under an inverted microscope for ballooning for up to 7 days. 12. Harvest tissue culture fluids typically on the 5th to 7th day of infection. 13. Centrifuge the infected cell culture supernatant at 1,500 rpm for 10 minutes to remove excess cells. 14. Gather the liquid that has risen to the top, and then divide it into cryovials. Add glycerol, a stabiliser, until the final concentration is 0.5 percent. Before using, divide viral stocks and keep them at a temperature of -80°C. 3.4 Japanese Encephalitis Virus Propagation JEV GP-78 strain, also known as the Japanese encephalitis virus Gorakhpur-78 strain, is a specific variant or isolate of the Japanese Encephalitis Virus. Japanese encephalitis is a flavivirus infection that is primarily transmitted by mosquitoes, particularly Culex species. This virus can cause Japanese encephalitis, a serious neurological disease that affects the brain. JEV GP-78 strain is neuroinvasive in nature has the ability to enter and infect the central nervous system, particularly spinal cord and brain. Neuroinvasive strains of the virus have a higher propensity to cause neurological symptoms and complications, including encephalitis. These complications can range from mild symptoms such as headaches and fever to more severe conditions, including paralysis, seizures, and cognitive impairments. Understanding the neuroinvasive nature of this specific strain, JEV GP-78, is essential for research and public health efforts because it helps scientists and healthcare professionals better comprehend the mechanisms of viral entry into the central nervous system. This knowledge can inform strategies for diagnosis, treatment, and prevention of Japanese encephalitis, particularly in regions where the disease is endemic. Furthermore, it highlights the significance of immunisation programmes and efforts to manage mosquito populations in order to decrease the prevalence of this catastrophic illness. The propagation and titration of the JEV (GP78 STRAIN) was performed using a plaque assay in Porcine Stable (PS) cells. Put 0.2x10⁵ cells/well of PS cells onto 12-well tissue culture plates. One hour after cell culture-grown JEV (GP78) virus has been serially diluted, infect PS cells. Rinse the cells with 1 X phosphate-buffered saline twice to remove any excess virus (PBS). Get a 2% combination of low-melting agarose and growth medium ready (1:1). After filling all of the wells, let the agarose overlay mixture set for 15 minutes at room temperature (RT). For three days at 37°C with 5% CO₂, let the cells develop. For one hour at 37°C, fix the cells with 4% formaldehyde. After 15 minutes at 37°C, stain the cells with a 1.5% crystal violet solution. Follow the steps above to wash the labelled cells three times with distilled water. Find the viral concentration in plaque-forming units (PFU) per millilitre by counting the plaques. To get the PFU value, divide the number of plaques (N), the viral dilution factor (DF), and the inoculum volume (V) by the total number of plaques (N-DF/V). 3.5 Rabies Virus Propagation The Rabies Virus CVS-11 strain, like other strains of the rabies virus, enters the CNS primarily through a process recognized as retrograde axonal transport. This process begins at the site of the initial infection, typically a peripheral site like a muscle or skin, where the virus gains entry via a bite from an infected animal. The virus then enters peripheral nerves and utilizes the neuronal transport system to travel along nerve fibers towards the CNS, ultimately reaching the spinal cord and brain. The virus is able to enter the central nervous system by retrograde axonal transport, which enables it to circumvent the blood-brain barrier (Sánchez-Martínez, and Juan C. de la Torre). 1. Retrieve the "Challenge Virus Standard Strain" (CVS)-11 of rabies virus from laboratory storage. 2. Propagate the virus in BHK-21 cells. 3. Grow BHK-21 cell monolayers in T75 flasks to 90% confluence. 4. Infect BHK cells with CVS-11 at a MOI (Multiplicity of Infection) of 0.8. 5. Harvest infected cells after 72 hours of infection. 6. Perform a total of three freeze-thaw cycles to disrupt cells. 7. Centrifuge the disrupted cells to collect virions. 8. Conduct the 50% TCID₅₀ (Tissue Culture Infectious Dose) assay on BHK-21 cells. 9. Seed BHK-21 cells in 96-well plates and allow them to grow overnight. 10. Infect cells with serially diluted virus samples. 11. Incubate infected cells for 5 to 7 days. 12. Examine plates under a microscope for cytopathic effects (CPE). 13. Calculate TCID₅₀ value using the Karber method formula. 3.6 Plaque Assay for Virus Titre Determination The Plaque Assay for Virus Titre Determination is pivotal in virology, enabling precise measurement of virus concentration. It plays a critical role in vaccine development, antiviral drug testing, and characterizing viruses. Additionally, it serves as a diagnostic tool in clinical settings and aids research into viral pathogenesis, contributing to our understanding of viruses and their impact on health and disease. 1. Put BHK-21 cells onto 12-well plates and let them grow into a monolayer. Incubate them overnight at 37 °C in a 5% CO₂ incubator. 2. To be sure the final plaque counts were accurate, inoculate the cell monolayer with a dilute virus culture supernatant. Leave to incubate for 90 minutes at 37 °C, stirring occasionally. 3. Remove the unattached virus and wash the cells with PBS after adsorption. 4. Spread 1 millilitre of solid culture media containing 2% FBS, DMEM, 1% Aquacide II, and 1% penicillin/streptomycin over the cells. 5. Plaques should be evident after 72 to 96 hours of incubation at 37 °C. 6. Allow the cell monolayers to fix for 2 hours at room temperature before staining them with crystal violet, which contains 3.7% methanol. 7. Then, carefully examine each well under a microscope to count the visible plaques. Plaques should appear as clear zones against the stained cell monolayer. Record the number of plaques in each well. 8. Take the average plaque count from all wells. Multiply this average by the dilution used during viral culture supernatant preparation. Divide this result by the volume of virus applied to the cell monolayer (usually in mL). Example Calculation for PFU/mL: If the average plaque count is 50 plaques, and you used a 10-fold dilution of the viral culture supernatant with a volume of 0.1 mL applied to the cell monolayer: Average plaque count: 50 plaques Dilution factor: 10 Volume of virus applied: 0.1 mL $\text{PFU/mL} = (\text{Average plaque count}) \times (\text{Dilution factor}) / (\text{Volume of virus applied})$ $\text{PFU/mL} = (50 \text{ plaques}) \times (10) / (0.1 \text{ mL}) = 50,000 \text{ PFU/mL}$ 9. Calculate the MOI (Multiplicity of Infection) by dividing the PFU/mL by the number of cells infected. Using a known cell density or

measuring the total number of cells in a well before infection may be used to calculate the number of infected cells. Example MOI Calculation: If you have 1 million cells in a well and the PFU/mL is 50,000: $MOI = (PFU/mL) / (\text{Number of cells infected})$
 $MOI = (50,000 \text{ PFU/mL}) / (1,000,000 \text{ cells}) = 0.05 \text{ MOI}$ 3.7 Virus infection on cells 3.7.1 Direct virus exposure on bEnd3 endothelial cells: The objective of this experiment is to investigate the direct infection of BBB endothelial cells, specifically bEnd3 cells, with JEV, DENV (Dengue virus), and Rabies virus (CVS-11). This S4 aims to simulate viral interactions with BBB endothelial cells, facilitating the study of potential neuroinvasive properties of these viruses. This experiment is significant as it allows researchers to assess the ability of JEV, DENV, and Rabies virus to infect BBB endothelial cells directly. Understanding how these viruses interact with the BBB is crucial for unraveling the mechanisms of viral neuroinvasion, which can lead to severe neurological diseases. Researchers can better understand virus-host interactions and find ways to avoid or lessen the severity of neuroinvasive infections by studying how viruses affect BBB endothelial cells. This experiment involves three different viruses, enabling a comparative analysis of their infection mechanisms and effects on BBB cells. Such comparative studies can reveal unique characteristics of each virus and aid in the development of targeted interventions. Findings from this experiment may contribute to the development of strategies to prevent or treat neuroinvasive viral infections, ultimately benefiting public health and enhancing our ability to combat diseases caused by JEV, DENV, and Rabies virus. 1. Use bEnd3 cells as the substrate for the BBB endothelial cell model, procured from ATCC- USA. 2. Gather the ingredients for the cell culture medium, which should include 7.5% sodium bicarbonate and 10% foetal bovine serum (FBS, Life Technologies, India). 3. Until confluence is achieved, keep the bEnd3 cells at 37°C with 5% CO₂. 4. Use mechanical disruption treatments or scraping to subculture the cells (Coming, India). 5. Centrifuge the subcultured cells at 1500rpm for 10 minutes. 6. Split the cells in the ratio of 1:2 to 1:4 as per the experimental requirements. 7. Plant bEnd3 cells in a 25 cm² flask and let them incubate in DMEM supplemented with 2% FBS for the night. 8. Primary infect the cells with Japanese encephalitis virus, Dengue virus, and Rabies virus (MOI = 0.1). 9. The National Institute of Virology in Pune, India, has the Dengue virus and the Japanese encephalitis virus (JEV GP-78, DENV2 #P23085). 10. Procure Rabies Virus Challenged Virus Strain (CVS-11) from CRI, Kausali. 11. Infect cells individually with JEV GP-78, DENV2, and CVS-11 in 5ml DMEM and 2% FBS. 12. Perform the virus infection for 2 hours in separate flasks and in triplicate. 13. Confirm the infection by qRT-PCR using SYBR-GREEN chemistry (Lancioti et al., 1992). 14. Perform the qRT-PCR using the Applied Biosystems Inc. StepOne Plus Machine. 15. Collect the supernatant for protein analysis. 16. Process the cells for extraction of transmembrane protein. 3.7.2 Brain endothelial cells exposed to supernatants of virus exposed RAW cell supernatant: The objective of this experiment is to investigate the immune response on brain endothelial cells and neuronal cells when exposed with supernatants of virus exposed murine monocyte (RAW) cell line. This study aims to understand how viruses, including JEV GP-78, DENV2, and CVS-11, can activate monocytes and subsequently affect the immune modalities and interaction dynamics with brain endothelial and neuronal cells. The significance of this experiment lies in its potential to elucidate the mechanisms of viral neuroinvasion and neuropathogenesis. By studying the virus-exposed monocytes effect on neuronal cells and brain endothelial cells, researchers can gain insights into the complex interplay between immune responses and viral infections in the CNS. This knowledge may contribute to a better understanding of diseases like Japanese encephalitis, dengue, and rabies, potentially leading to the development of therapeutic strategies targeting neuroinvasive viruses. 1. Use the Murine monocyte (RAW) cell line as the model for the immune response stimulus. 2. Infect RAW cells with JEV GP-78, DENV2, and CVS-11 (MOI = 0.1). 3. Perform the virus infection for 2 hours individually with JEV GP-78, DENV2, and CVS-11 in separate flasks and in triplicate. 4. Collect the virus-activated RAW cell supernatant post-infection. 5. Overlay the virus-activated RAW cell supernatant on bEnd3 cells and neuronal-like SH-SY-5Y and N2a cell lines. 6. Expose the bEnd3 cells using the virus-activated monocyte supernatant after 2 hours of direct infection, incubating for a further 2 hours. 7. Propagate an identical set of control flasks. 8. Confirm the infection by RT-PCR using SYBR-GREEN chemistry (Lancioti et al., 1992). 9. Perform qRT-PCR using the Applied Biosystems Inc. StepOne Plus Machine. 10. Collect the supernatant from bEnd3 and neuronal cells for protein analysis. 11. Process the cells for the extraction of transmembrane proteins. 3.8 MTT Cytotoxicity Assay The objective of the MTT Cytotoxicity Assay is to assess the cytotoxic effects of JEV GP-78, DENV2, and CVS-11 viruses on various cell types, including RAW, bEnd3, SH-SY-5Y, and N2a cells, with the aim of understanding their potential role in disrupting blood-brain barrier tight junction proteins. By inoculating these viruses into different cell lines and measuring cytotoxicity, this experiment seeks to elucidate the impact of these viruses on cell viability, which may provide insights into their contribution to neuropathogenesis. The significance of this experiment lies in its ability to provide quantitative data on the cytotoxicity induced by these neurotropic viruses in relevant cell models. Understanding how these viruses affect cell viability is essential for unraveling the mechanisms behind blood-brain barrier disruption and subsequent neuropathogenesis. This information may inform strategies for mitigating the neurological complications associated with viral infections, ultimately advancing our knowledge of virus-host interactions in the context of the blood-brain barrier. 1. Inoculate JEV GP-78, DENV2, and CVS-11 viruses at an MOI of 0.1 into RAW, bEnd3, SH-SY-5Y, and N2a cells in separate 96-well plates. 2. Maintain identical plates for JEV GP-78, DENV2, and CVS-11 virus-infected RAW cells for infection with supernatants of virus-exposed RAW cells. Use DMSO as negative control. 3. For 16 to 18 hours, incubate the plates at 37°C with 5% CO₂. 4. To every well, add 100µl (10 percent) of a 5mg/ml MTT solution. 5. Incubate the plates for an additional 4 hours at 37°C. 6. Add DMSO to every well to dissolve the formazan crystals. 7. Fill out the Multimode ELISA reader with the sample and read the absorbance at 550 nm. Fig 3 MTT Assay mechanism for determining cytotoxicity Source credit: [Ghasemi, Mahshid et al.](#) "The MTT Assay: Utility, Limitations, Pitfalls, and Interpretation in Bulk and Single-Cell Analysis." [International journal of molecular sciences vol. 22,23 12827. 26 Nov. 2021. doi:10.3390/ijms222312827](#). 3.9 Cells RNA extraction The respective viral RNA was extracted from cell culture supernatants harvested after successful virus propagation. Viral RNA Kit (Himedia, India) was used for the RNA extraction of respective viruses. To conclude, the lysis and extraction of 106-108 cells were carried out according to the manufacturer's methodology. Thermo Scientific of India reverse-transcribed the material to cDNA, which was then kept at -80°C until additional research was performed to assess gene expression. 3.10 Virus infected cells RNA isolation The objective of this experiment is to isolate and purify viral RNA from infected cells, clinical specimens, and viral isolates using the QIAamp® Viral RNA Mini-isolation kit. This purified viral RNA will then be used for various molecular assays, including qRT-PCR and RT-PCR, to study the role of viruses in disrupting blood-brain barrier tight junction proteins, ultimately contributing to our understanding of neuropathogenesis. This RNA isolation process is a critical step in elucidating the molecular mechanisms underlying blood-brain barrier disruption by viruses. By isolating viral RNA, researchers can analyze the expression of cytokine genes and other factors involved in neuropathogenesis, providing valuable insights into the pathophysiology of neurological complications caused by viral infections. Additionally, this protocol ensures the availability of high-quality viral RNA for further studies, including the development of potential therapeutic interventions targeting virus-induced blood-brain barrier disruption. 1. The QIAamp® Viral RNA small isolation kit was used to extract total RNA from all cells infected with the virus (Qiagen, India). 2. To simplify, the lysis and extraction of 106-108 cells were carried out according to the manufacturer's methodology. 3. Following the directions on the QIAamp® Viral RNA micro kit (QIAGEN GmbH, Hilden, Germany), the RNA was manually extracted. QIAamp Viral RNA kit provides the fastest and easiest way to purify viral RNA for dedicated use in amplification technologies. 4. Utilizing centrifuge or vacuum technologies, the kit takes use of the characteristics of a membrane based on silica gel. 5. To isolate intact viral RNA, the sample is first lysed under very denaturing conditions to render RNases inactive. 6. The next step is to fine-tune the buffering conditions such that the RNA binds optimally to the membrane that is based on silica gel. 7. Two distinct wash buffers are used to wash contaminants once the RNA attaches to the membrane. 8. Eluted from the high-quality pure RNA was a specialised RNase-free buffer that did not include any nucleases, proteins, or other contaminants or inhibitors. 9. The RNA was extracted from the clinical specimens and cell culture supernatant of virus isolates. Following the instructions of manufacturer, the RNA extraction process was carried out. 10. Preparations of reagents: Carrier RNA: 310 µl of buffer AVL was added to 310 µg lyophilized RNA to obtain a solution of 1µg/µL. Ready to use AVL: 5.6µl of carrier RNA was added to per 560 µL of AVL just before use. Buffer AW1: The supplied 95 mL concentrate was diluted with 125 mL ethanol (96-100%). Buffer AW2: The supplied 250 mL concentrate was diluted with 160 mL ethanol (96-100%). 11. The 1.5 mL micro centrifuge tubes were filled with buffer AVL that contained carrier RNA at a concentration of 1 µg/µL. After thawing, 140 µL of the viral isolates and clinical specimens were added and left to incubate at room temperature. 12. After adding 560 µL of ethanol (96-100 percent), the sample was thoroughly mixed using pulse vortexing for 15 seconds before being centrifuged. 13. The next step was to put 630 µL of the sample onto the spin column's membrane. The membrane was then placed in a 2 mL collecting tube and spun at 8,000 rpm for 1 min. 14. Afterwards, the filtrate-containing tube was removed and a fresh tube was put in its place. The membrane was then treated with 500 µL of buffer AW1 and centrifuged at 8000 rpm for 1 min. 15. Every stage

included disposing of the collecting tube. In addition, 500 µL of Buffer AW2 was spun in a centrifuge at 14,000 rpm for 3 mins after being applied to the previously mentioned membrane. 16. Next, a clean micro-centrifuge tube was used to put the spin column. The membrane was then treated with 60 µL of elution buffer AVE. After incubating at room temperature for 1 minute, the mixture was centrifuged at 8,000 rpm for 1 min. 17. In the 60 µL that was eluted, the virus RNA was present. While the rest of the RNA was kept in a deep freezer at -80°C, the eluted RNA was used for qRT-PCR and RT-PCR. This was then subjected to reverse transcription using cDNA from Thermo Scientific in India and kept at a temperature of -80°C until the cytokine gene expression analysis was completed. 3.11 Gene Expression studies The objective of the gene expression study is to examine the impact of virus infection on various categories of genes, including inflammatory, regulatory, BBB breakdown, and reactive oxygen species (ROS) generation genes. This experiment aims to understand how viruses influence the expression of these genes, particularly those related to BBB integrity, and their potential role in disrupting tight junction proteins within the BBB. By quantifying gene expression changes using Real-time RT-PCR and evaluating fold changes, this research seeks to explain the molecular mechanisms underlying virus-induced neuropathogenesis. The significance of this experiment lies in its potential to reveal the molecular pathways through which viruses disrupt the blood-brain barrier and compromise neurological function. Understanding the modifications in gene expression, especially in genes related to BBB breakdown, can provide valuable insights into the pathogenesis of neurological diseases caused by viral infections. This finding may contribute to the development of targeted interventions to protect and keep the integrity of the BBB, ultimately improving our ability to combat neuropathogenesis associated with viral infections. Three sets of gene expression were classified based on their functions: (i) Inflammatory genes, including interleukin-18 (IL-18), *interferon gamma* (IFN-γ), *interleukin-10* (IL-10), *tumor necrosis factor alpha* (TNF-α), and interleukin-1 beta (IL-1β), (ii) Regulatory genes, such as IL-6 ("Interleukin-6") and MCP-1 ("Monocyte Chemoattractant Protein-1"); (iii) BBB breakdown genes, including occludin, matrix metalloproteinases (MMP2, MMP9), and TIMP1 (Tissue Inhibitor of Metalloproteinase); and (iv) Reactive oxygen species (ROS) generation genes, such as nNOS (Neuronal Nitric Oxide Synthase), eNOS (Endothelial Nitric Oxide Synthase), and iNOS (Inducible Nitric Oxide Synthase). A RealTime RT-PCR kit (Thermoscientific, India) was used to amplify the genes. The primer and cycling conditions were utilized as defined elsewhere (Overbergh et al., 2003; Chien et al., 2011). The housekeeping gene GAPDH was used for normalization. The value of ΔCt for each gene was computed using three separate wells of all cell lines that were directly and indirectly infected with the respective viruses. To determine the fold change, the ΔΔCt value was calculated using the mean ΔCt value. A change was considered significant when the ΔΔCt value was more than 2 (Schmittgen and Livak, 2008). Table 1 cDNA preparation using RevertAid First strand cDNA synthesis kit: Reaction Components Reaction Volume Template RNA 100 ng Primer 1 µL NF water Upto 11 µL 5X reaction buffer 4 µL 10mM dNTP Mix 2 µL Enzyme(200U/µL) 1 µL Total 20 µL Table 2 cDNA preparation temperature condition of thermal cycler: Settings Temperature Time Step 1 25°C 5 mins Step 2 42°C 60 mins Step 3 70°C 5 mins Step 4 4°C ∞ Table 3 Primers Sequence for in-vitro BBB disruption qPCR analysis: Gene Primer Sequence Annealing Temperature Amplicon size MMP-2-F 5'-TTCCCTAAGCTCATCGACAGT-3' 55°C 196 bp MMP-2-R 5'-CACGCTCTTGAGACTTTGGTCT-3' MMP-9-F 5'-GCGTGTCTGGAGATTGCACTT-3' 55°C 229 bp MMP-9-R 5'-TATCCACGCGAATGACGCT-3' iNOS-F 5'-GACATTACGACCCCTCCAC-3' 55°C 254 bp iNOS-R 5'-GCACATGCAAGGAAGGAAAC-3' TIMP-1-F 5'-GCAACTCGGACCTGGTCATAA-3' 55°C 226 bp TIMP-1-R 5'-CGGCCGTGATGAGAACT-3' IL-6-F 5'-GAGGATACCACTCCCAACAGACC-3' 56°C 221 bp IL-6-R 5'-AAGTGCATCATCGTTTTCATACA-3' MCP-1-F 5'-CTTCTGGGCTGCTGTTC-3' 55°C 285 bp MCP-1-R 5'-CCAGCCTACTCATTGGGATCA-3' Occludin-F 5'-CACACTTGTCTGGACAGAGG-3' 56°C 197 bp Occludin-R 5'-TGAGCCGTACATAGATCCAGGAGC-3' IL-10-F 5'-GGTGTGCCAAGCCTTATCGGA-3' 55°C 191 bp IL-10-R 5'-ACCTGTCTCCACTGCCTTGTCT-3' IL-18-F 5'-ACTGTACAACCGCAGTAATACGG-3' 58°C 271 bp IL-18-R 5'-AGTGAACATTACAGATTATCCC-3' IL-1β-F 5'-CAACCAACAAGTGATATTTCTCCATG-3' 55°C 245 bp IL-1β-R 5'-GATCCACACTCTCCAGCTGCA-3' TNF-α-F 5'-CATCTTCTCAAAATTCGAGTGACAA-3' 55°C 242 bp TNF-α-R 5'-TGGGAGTAGACAAGSTACAACCC-3' IFN-γ-F 5'-TCGCCGCGATCAAGGAGTTCTTCGGC-3' 58°C 201 bp IFN-γ-R 5'-TGACGTGCGGACCTCCACGGCGAC-3' GAPDH-F 5'-GCACAGTCAAGGCCGAGAAAT-3' 55°C 211 bp GAPDH-R 5'-GCCCTTCTCCATGGTGGTGAA-3' Table 4 Temperature conditions for in-vitro BBB disruption qPCR analysis: CYCLE STEP TEMPERATURE TIME CYCLES Initial Denaturation 95°C 30 secs 1 Denaturation Annealing Extension 95°C 55°C 72°C 5 secs 30 secs 30 secs 30 secs (* plate read) 40 Melt Curve 60-95°C various 1 *Optics were kept open. #Annealing temperature as mentioned in above Primer sequence Table No (B) In-vivo studies in Drosophila melanogaster glial-brain barrier 3.12 Drosophila breeding, maintenance Incubators at temperatures between 18 and 29 degrees Celsius are used to keep flies in vials with covers on them. It is also possible to store certain fly stocks on benches or shelves at room temperature. Moving fly vials from one room to another can be required. Stoppers and bottles/vials need to be fastened securely in their respective racks while this is being done. Secondary containment should also be used, e.g., placing racks inside a plastic bag or box. 3.13 Drosophila cross setup Before starting with crossing genetic fly lines with reporter lines. We crossed the unbalanced fly lines with GFP, Pr, Tm6B, and Sb markers. The ideal breeding temperature for flies is at 25°C but UAS-mac-NOS lines weren't producing any correct marked progenies. Therefore, we tried breeding them at 18°C, through which we could obtain correct transgenic progenies from some of the crosses. 1. Crosses for up-regulation of NOS: Table 5 Crosses between genetic and reporter lines of Drosophila for up-regulation of NOS Type of Glia Genetic lines Reporter line All glia repo-G4 (precursor process need balancer) Perineurial glia w-; NP6293-Gal4; UAS- GFP/CyO w-; S/CyO; UAS-mac-NOS/Tm6B Sub-perineurial Moody-G4 (precursor process glial need balancer) 2. Crosses for down-regulation of NOS: Table 6 Crosses between genetic and reporter lines of Drosophila for down-regulation of NOS Type of Glia Genetic lines Reporter line All glia repo-G4 (precursor process need balancer) Perineurial glia w-; NP6293-Gal4; UAS- GFP/CyO UAS-NOS-RNAi #50675 Sub-perineurial Moody-G4 (precursor process glial need balancer) 3.14 Drosophila fly brain RNA extraction 1. In this experiment, gene expression studies were conducted using fruit fly heads. A total of 50-55 heads were dissected for each transgenic correct progeny. 2. The heads were immediately processed for RNA extraction using the NEB Monarch RNA isolation kit. 3. The manufacturer-provided methodology was followed to conduct the RNA extraction. 4. The NEB Monarch RNA isolation kit was used to extract RNA from the dissected fly heads. The kit's technique called for first lysing the brain tissue using a lysis buffer, and then, in the presence of a highly concentrated chaotropic salt, to bind the RNA to a silica membrane. 5. After washing the membrane to remove contaminants, RNA was eluted from the membrane with a low salt elution buffer. 6. The RNA was then subjected to quality control and quantification using a spectrophotometer. The resulting RNA samples were used for downstream gene expression studies. 3.15 Drosophila fly brain gene expression Table 7 cDNA preparation using First strand cDNA synthesis: Reaction Components Reaction Volume 10X reaction buffer 2 µL 25X dNTP Mix (100mM) 0.8 µL 10X RT Random Primer 2 µL NF water Upto 4.2 µL Enzyme(20U/µL) 1 µL 10 µL Add the above component on an ice-chiller in the given order to make 10 µL of 2X master mix. To this add 10 µL of extracted RNA samples in respective tubes. Mix gently and centrifuge the reaction tubes before putting in thermal cycler. For random hexamer primers 5 mins at 25°C followed by 37°C for 60 mins. Stop the reaction at 70°C for 5 mins. Use it immediately for qPCR step or store at -20°C for 1 week. Table 8 Primer Sequence for gene expression studies in Drosophila Gene Primer Sequence Annealing Temperature Amplicon Size iNOS-F 5'-ACCAGACATCCCATGTGAAGC-3' 56°C 218 bp iNOS-R 5'-GCCTGTGTAATGGAACCATACCA-3' dmMMP1-F 5'-TCAGTGTTCATAGTCGTAGGCA-3' 56°C 159 bp dmMMP1-R 5'-GGTAGATAGCCGAATCTGGGAC-3' dmMMP2-F 5'-AAAACCCGCTATCTCCGAGGA-3' 56°C 148 bp dmMMP2-R 5'-TGTAATGTTGCCAAAGACTGGA-3' Moody-F 5'-TATCATCGACCTCTGTATTGCCG-3' 56°C 150 bp Moody-R 5'-AGGGATACCCCTATGTTTCCG-3' NrXIV-F 5'-CAACTCCGATGGCAACTCTATC-3' 56°C 124 bp NrXIV-R 5'-CCACACGATGGAATACGAT-3' Table 9 cDNA preparation temperature condition for Drosophila model: Settings Temperature Time Step 1 25°C 5 mins Step 2 37°C 2 x 60 mins Step 3 85°C 5 mins Step 4 4°C ∞ 3.16 Gene expression studies on Drosophila fly brain extracted RNA Prepare cDNA of interest utilizing desired DNA extraction as well as purification technique. Run each cDNA standard and sample in triplicate as per the kit protocol. Table 10 Luna@ Universal qPCR Master Mix Protocol for in-vivo BBB disruption analysis: COMPONENT 20 µL REACTION FINAL CONCENTRATION Luna Universal qPCR Master Mix 10 µL 1X Forward primer (10 µM) Reverse primer (10 µM) 0.5 µL 0.5 µL 0.25 µM Template cDNA variable < 100 ng Nuclease-free Water to 20 µL Table 11 Thermal cycling conditions for gene expression studies in Drosophila: 69 CYCLE STEP TEMPERATURE TIME CYCLES Initial Denaturation 95°C 30 secs 1 Denaturation Annealing Extension 95°C 56°C 72°C 5 secs 30 secs 1 min (* plate read) 40 Melt Curve 60-95°C various 1 *Optics were kept open 3.17 Immunological studies nrX4, moody-GFP protein studies The procedure for Drosophila brain dissection and immunostaining is defined in details in the following papers: 1) A protocol for dissecting Drosophila melanogaster brains for immunostaining or live imaging (2006) Nat. Protoc. 1, 2110–2115. 2) Improved and expanded Q-system reagents for genetic manipulations (2015) Nature Methods 12, 219–222. Confocal Microscopy Protocol for GFP-Fly

Brain: A) Processing of sample (Fly Brain) B) Imaging of fixed mounted sample (A) Fly Brain sample processing: DAY 1 Dissect the brain in chilled 1X PBS (For each brain it should take less than 6-10 mins). Remove the trachea, and optical parts (orange eye) to make the brain as clean as possible. Put the brain directly in (approximately 200-500 μ L) 4%PFA in PBT freshly prepared at room temperature or freshly thawed a frozen vial of PFA. Do not try to freeze the vial again for further use. Once the vial is thawed it should be used directly. Keep this (Brain+PFA) vial on a shaker for a sharp 20 minutes only for fixation at room temperature, dark (Cover with a black opaque tip box or something). After 20 mins, wash the brains with PBT for 5-6 hours. Remove the 4%PFA and immediately add PBT (approximately 200-500 μ L) and keep it on the shaker for 30 mins, 35 rpm at room temperature, dark. After 30 mins remove PBT add fresh PBT and keep the vials on the shaker. Repeat this procedure 3-4 times. Block fly brain in 5%NGS in PBT for at least 30 mins at room temperature. Eliminate the final PBT and add approx. 200ul of Blocking solution in each vial and let it incubate at room temperature for 30 mins, 35 rpm, dark.

Table 12 Preparation of Blocking solution for confocal microscopy of Drosophila BLOCKING SOLUTION NGS 5% 5 μ L 30 μ L PBT Up to 95 μ L 570 μ L 100 μ L 600 μ L After blocking for 30 mins, place the brain in Primary Antibody mix at 4°C (in a cold room) for three nights. The primary antibody Mouse nc82 stains the Brp protein (Bruchpilot – German for Crash Pilot) in red. The Brp protein is ubiquitously expressed in the Drosophila brain and more localized to the neuropile region (Knapik, Stephan, et al., 2009). While the Rabbit anti- 71 GFP primary antibody stains the moody protein in the sub-perineural glia barrier of the Drosophila brain in fluorescence green. Table 13 Protocol for the preparation of Primary Antibody PRIMARY ANTIBODY MIX Mouse nc82 (DSHB, 1:25) 4% 4 μ L 24 μ L Rabbit anti-GFP (Life Technologies # A11122, 1:100) 1% 1 μ L 6 μ L NGS 5% 5 μ L 30 μ L PBT Up to 90 μ L 540 μ L 100 μ L 600 μ L DAY 4 Wash brains in PBT for many hours at room temperature. Remove the Primary antibody mix add PBT (approximately 200-500 μ L) and keep it on the shaker for 30 mins, 35 rpm at room temperature, dark. After 30 mins remove PBT add fresh PBT and keep the vials on the shaker. Repeat this procedure 3-4 times. Remove the PBT in the last wash and add (approximately 200- 500 μ L) of Secondary Antibody Mix for 2 nights (Day 4- Day 5) at 4°C (in a cold room). Table 14 Protocol for the preparation of Secondary Antibody SECONDARY ANTIBODY MIX Alexa 488 anti-rabbit (Invitrogen #A11034) 0.5% 0.5 μ L 3 μ L Cy3 anti-mouse (Jackson ImmunoResearch #115-165-062) 0.5% 0.5 μ L 3 μ L NGS 5% 5 μ L 30 μ L PBT upto 94 μ L 564 μ L 100 μ L 600 μ L DAY 6 Wash brains in PBT for many hours at room temperature. Remove the Secondary antibody mix add PBT (approximately 200-500 μ L) and keep it on the shaker for 30 mins, 35 rpm at room temperature, dark. After 30 mins remove PBT add fresh PBT and keep the vials on the shaker. Repeat this procedure 3-4 times. Place the brain in a mounting solution (Slow Fade Gold) overnight at 4°C (in a cold room). DAY 7 Mount brains on a microscopic slide. For imaging these slides follow the below-mentioned protocol. (B) Imaging of fixed mounted sample: The SMC 2009 and Power Supply should be turned on, and the System Switch should be turned on. The CPU should be turned on, and the user should log on as an LSM user. The Components Switch should be turned on, and the Laser Key should be turned on. The Zen Blue 2.6 System Software should be started, and the +Zen System should be selected. For sample imaging, the Magnification Lens (Airy Green 20X) should be selected, the Top button should be pressed for Stage Up, and the Bottom Button should be pressed for Stage Down. The slide should be placed, 73 and the rod should be pushed in to see with the eye lens or pulled out to see the LSM confocal image. Locate should be clicked, and Transmitted Light/GFP/RFP should be selected. Then, the brain sample should be focused on, and the GFP/DAPI filter should be clicked. The sample (brain) should be checked again, and it should be focused. The acquisition should be clicked, and Smart Setup should be clicked. The experiment should be configd, and smartest Line should be selected, and OK should be clicked. Channels should be clicked, Track 1 on Imaging Setup should be clicked, and EGF [1AU] should be clicked. Live should be clicked, and Acquisition Mode should be set to Frame. The area should be cropped or scanned, and the image should be realigned on the screen. Pixels (Pre-set) such as 128x128, 256x256, or Optimal (e.g., 5172x5172) should be selected. The scan speed should be set to LOWEST or HIGHEST, and the direction should be set to Uni or Bi. Averaging should be set to 0[2x]4x], and bits per pixel should be set to 8 or 16. For display, [Min/Max] should be clicked, and the Range Indicator should be clicked to decrease the master gain (v) and decrease the red dots. The Laser Power should be kept constant at 0.2%, and the gain should be increased to 530-750V. For the Z stack, First should be set by pressing Ctrl and scrolling up & down with the mouse until the beginning of the top edge of the brain is reached. The last should be set by pressing Ctrl and keeping scrolling up & down with the mouse until the beginning of the bottom end of the brain is reached. Optimal Slicing or Manual Setup can be chosen, and Start Experiment should be clicked. After completing the Z-Experiment, Gallery should be clicked, and 3D should be clicked to view the 3D images. Save Image should be clicked. For Tile, X-1 and Y-3 should be selected, and X1 (only one x-axis plane) should be clicked to start the experiment. For processing, Stitching should be clicked, Fuse should be clicked, and ALWAYS Apply in [NEW OUTPUT] file should be clicked. The NewOutput stitched file should be saved in .tiff format. 3.18 Transmission Electron Microscopy of Drosophila fly brain The protocol for the Transmission electron microscopy experiment on Drosophila fly brains is as follows dissect at least 6-10 brains from each of the following groups: Moody-NOS-RNAi, Moody-NOS-NOS, Moody-UAS-mac-NOS, and Moody-Parent the BBB. 1. Rapidly dissect brains into cold fly saline, remove any air sacs to prevent the brains from floating, and place the brains in 1X chilled PBS on a chilled watch glass. 2. Remove air sacs, trachea, and possibly the orange optical portion of the fly eye to prevent the brains from floating and entangling with the trachea. 3. Fix the brains by immersing them in 2% paraformaldehyde + 2.5% glutaraldehyde in 0.1M sodium cacodylate at 4°C for 1 hour, followed by 1 hr at room temperature. 4. After dissection, immediately transfer each brain to a 10ml glass vial containing fixative solution. 5. Do not keep the glass vials on ice, let them remain at room temperature until all the fly brains are dissected. 6. Place the glass vials on a 360° rotator and let them incubate at room temperature for 1 hour, keeping the rotation speed at 4rpm. 7. Rinse the brains in 0.1M sodium cacodylate 3 times for 5 mins each time, using glass pipettes/droppers to give any subsequent washes. 8. Remove the fixative solution without drying up the brain and add 0.1M sodium cacodylate solution, then place it back on the rotator for washing at 4rpm. 9. Post-fix the brains with 1% OsO4 in 0.1M sodium cacodylate comprising 0.1M immediately after removing the last wash of 0.1M sodium cacodylate. Ensure that the volume is sufficient to immerse all the brains well. 10. Incubate the fly brains in an Osmium fixative solution for 1 hour on a rotator machine rotating at 4rpm. 11. View the sections using a transmission electron microscope, and capture images of the Moody-NOS-RNAi, Moody-NOS-NOS, Moody-UAS-mac-NOS, and Moody-Parent the BBB groups of Drosophila fly brains. Fig 4 Flow method for fixation and embedding of Drosophila fly brain for TEM imaging Chapter 4 Results 4.1 In silico studies of the dengue envelope protein Dengue Envelope Protein (DEP) structure construction (SWISSMODEL) Fig 5 Dengue Envelope Protein (DEP, 3j27.1.C) obtained from SWISS-MODEL After submitting the Dengue envelope protein sequence in SWISS-MODEL software, we obtained the above energy-minimized stable structure. The PATCHDOCK and FIREDOCK software packages were used to further submit this structure to protein-protein interaction with brain membrane proteins. The binding energy obtained after PATCHDOCK and FIREDOCK are mentioned in the below table. Table 15 The comparison of the lowest energy pose of Dengue envelope protein with other viruses mentioned in the literature. Receptor Binding Energy for DEP (kJ/mol) Reported Binding Energy (kJ/mol) Voltage-dependent P-Q type calcium channel subunit alpha 1A -17.26 -53.12 VLPAC 13C -49.60 -45.13 Cytoplasmic dynein 1 heavy chain 1 -45.12 -50.67 Plasma lemma vesicle-associated protein -38.72 -29.27 Nesprin1 -36.31 -44.73 Brefeldin-A inhibited guanine nucleotide exchange protein-2 -36.15 -136.18 Annexin A6 -24.81 -19.56 Transcription factor SOX-5 -23.95 -50.81 Rabenosyn-5 -21.00 -84.19 *The lowest binding free energy found in the literature of different viruses such as WNV, JEV, and other Flaviviruses Fig 6 In-silico virus envelope protein- brain endothelial transmembrane receptor interaction analysis (a) DEP-ANXA6 Annexin A6 brain membrane receptor interactome (b) DEP-VP13Cvacuolar protein sorting associated protein-13Cbrain membrane receptor interactome (c) PLVAP Plasma lemma vesicle-associated protein brain membrane receptor obtained from FireDock server. 4.2 Virus Propagation 4.2.1 Dengue virus Following Fig7 shows control (uninfected) C6/36 cells and DENV infected C6/36 cells. Mock-infected C6/36 cell line DENV-infected C6/36 cell line Fig 7 C6/36 cell lines uninfected and infected with Dengue virus type 2 The TCID50-CPE titre for the DENV-2 serotype was found to be 10 / 100ul. 5 4.2.2 Japanese Encephalitis Virus Porcine-stable cell lines were overlaid with JEV GP-78 virus with 2% Fetal Bovine Serum with a control mock-infected flask. After completion of the incubation period, the cell clusters were observed indicating the alleviation in virus replication in cells (can be observed in the below image). Fig 8 Porcine Stable cell lines uninfected and infected with JEV-GP78 virus 4.2.3 Rabies Virus Post 24-96 hours after incubation of Rabies Virus (CVS-11) propagation in BHK-21 cells was observed. BHK-21 infected with CVS after 24 hrs BHK-21 infected with CVS after 48 hrs BHK-21 infected with CVS after 72 hrs BHK-21 infected with CVS after 96 hrs Fig 9 BHK21 cell lines uninfected and infected with CVS-11 rabies virus 4.3 Cytotoxicity Analysis of virus on cells: The viral apoptosis upon direct infection with JEV GP-78, DENV2, and CVS-11 and with supernatants of virus-exposed murine monocytic cells on brain endothelial cell lines (bEnd3) and neuronal cell lines (SH-SY-5Y and N2a). The cytotoxicity assay was analyzed for bEnd3 exposed to supernatants from

individual virus-activated murine monocyte cells (RAW) post 2 hours of infection. The dimethyl sulfoxide (50 uM) [was kept as a control in all the groups](#) of experiments. Fig 10 Percentage cytotoxicity observed on bEnd3, N2a, and SH-SY-5Y cells when infected with JEV GP-78, DENV2, and CVS-11. 4.4 Gene expression analysis of In-vitro studies on bEnd3 cells The bEnd3 cells were harvested post-exposure with JEV GP-78, DENV2, CVS-11, and the supernatant from these viruses exposed to murine monocytes. Then, total RNA was extracted to confirm the extent of disruption of BBB tight junction proteins that might be caused due to the alleviation of inflammatory cytokines. The gene expression analysis was carried out using Applied Biosystems Inc. GAPDH was kept as an endogenous reference 82 control. The mean fold change and the standard deviation are mentioned below. This test is appropriate for comparing means across multiple groups (in this case, different viruses) simultaneously. It helps identify whether the means of gene expression levels are statistically different and if the variations observed are due to exposure to the respective viruses. The results of the statistical analysis revealed significant variations [in the expression levels of each gene when compared to the control group](#). This suggests that exposure to different viruses indeed influences the gene expression profiles in bEnd3 cells. The means $\pm$ SEMs from three independent replicates were calculated for each gene, providing a comprehensive understanding of the impacts of viral exposure on the expression of these crucial genes. IFN γ ns 10 Mean Fold Change DENV 8 DENV+RAW/ B3 6 JEV JEV+RAW/ B3 4 RABV 2 RABV+RAW/ B3 0 V B 3 V V B 3 D E N W / J E / B 3 A W R A B W / N V + R A E V + R B V + R A D E J R A Fig 11 Expression of IFN-gamma in the bEnd3 cells post-exposure to different viruses. _ Table 16 Gene expression of IFN-gamma after virus exposure to bEnd3 cells Group Mean Fold Change DENV 3.46 DENV+RAW/B3 6.34 JEV 2.66 JEV+RAW/B3 8.43 RABV 1.79 RABV+RAW/B3 2.66 IL-6 ns ns 8 DENV Mean Fold Change 6 DENV+RAW/ B3 JEV 4 JEV+RAW/ B3 RABV 2 RABV+RAW/ B3 0 V B 3 V A B V B 3 D E N W / J E / B 3 A W R W / N V + R A E V + R B V + R A D E J R A Fig 12 Expression of IL-6 in the bEnd3 cells post-exposure to different viruses. _ Table 17 Gene expression of IL-6 after virus exposure to bEnd3 cells Group Mean Fold Change DENV 6.01 DENV+RAW/B3 5.19 JEV 6.19 JEV+RAW/B3 6.84 RABV 0.95 RABV+RAW/B3 2.93 15 Mean Fold Change 10 5 IL-10 ns 0 V B 3 V V B 3 D E N W / J E / B 3 A W R A B W / V + R A E V + R B V + R A D E N J R A Fig 13 Expression of IL-10 in the bEnd3 cells post-exposure to different viruses. _ Table 18 Gene expression of IL-10 after virus exposure to bEnd3 cells Group Mean Fold Change DENV 1.54 DENV+RAW/B3 12.74 JEV 0.12 JEV+RAW/B3 2.83 RABV 0.03 RABV+RAW/B3 1.73 IL1 β 25 [Mean Fold Change 20 15 10 5 0](#) V B 3 V V B 3 D E N W / J E / B 3 A W R A B W / V + R A E V + R B V + R A D E N J R A Fig 14 Expression of IL-1 β in the bEnd3 cells post-exposure to different viruses. _ Table 19 Gene expression of IL-1 β after virus exposure to bEnd3 cells Group Mean Fold Change DENV 6.36 DENV+RAW/B3 8.4 JEV 1.51 JEV+RAW/B3 20.06 RABV 0.04 RABV+RAW/B3 2.52 IL18 50 ns Mean Fold Change 40 30 20 10 0 V B 3 V V B 3 D E N W / J E / B 3 A W R A B W / N V + R A E V + R B V + R A D E J R A Fig 15 Expression of IL-18 in the bEnd3 cells post-exposure to different viruses. _ Table 20 Gene expression of IL-18 after virus exposure to bEnd3 cells Group Mean Fold Change DENV 35.09839935 DENV+RAW/B3 38.42 JEV 13.45434264 JEV+RAW/B3 22.11536 RABV 5.809175169 RABV+RAW/B3 7.41261 INOS 20 ns Mean Fold Change 15 10 5 0 V B 3 V V B 3 D E N W / J E / B 3 A W R A B W / N V + R A E V + R B V + R A D E J R A Fig 16 Expression of INOS in the bEnd3 cells post-exposure to different viruses. _ Table 21 Gene expression of INOS after virus exposure to bEnd3 cells Group Mean Fold Change DENV 6.46 DENV+RAW/B3 14.8 JEV 4.95 JEV+RAW/B3 8.89 RABV 1.20 RABV+RAW/B3 1.76 MCP-1 8 ns Mean Fold Change 6 4 2 0 E V B 3 3 D E N W / B 3 J J W / B V R A W / B V + R A + R A + R A D E N J E V B V R A DENV JEV RABV DENV+RAW/ B3 JEV+RAW/ B3 RABV+RAW/ B3 Fig 17 Expression of MCP-1 in the bEnd3 cells post-exposure to different viruses. The outcomes reflect the means $\pm$ SEMs from three different replicates (Asterisks represent significant differences, compared to control.; [ns, not significant](#)* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.) Table 22 Gene expression of MCP-1 after virus exposure to bEnd3 cells Group Mean Fold Change DENV 1.55 DENV+RAW/B3 4.59 JEV 1.26 JEV+RAW/B3 6.02 RABV 0.74 RABV+RAW/B3 3.03 MMP-2 ns 25 [Mean Fold Change 20 15 10 5 0](#) N V / B 3 J E V B 3 3 D E W W / B V / B + R A R A R A W + R A D E N J E V + B V R A Fig 18 Expression of MMP-2 in the bEnd3 cells post-exposure to different viruses. _ Table 23 Gene expression of MMP-2 after virus exposure to bEnd3 cells Group Mean Fold Change DENV 14.55 DENV+RAW/B3 22.95 JEV 4.38 JEV+RAW/B3 6.78 RABV 1.22 RABV+RAW/B3 1.45 MMP-9 20 ns ns Mean Fold Change 15 10 5 0 V B 3 V V B 3 D E N W / J E / B 3 A W R A B W / V + R A E V + R B V + R A D E N J R A Fig 19 Expression of MMP-9 in the bEnd3 cells post-exposure to different viruses. _ Table 24 Gene expression of MMP-9 after virus exposure to bEnd3 cells Group Mean Fold Change DENV 13.89 DENV+RAW/B3 12.27 JEV 4.34 JEV+RAW/B3 7.92 RABV 1.32 RABV+RAW/B3 1.21 Occludin 10 ns ns Mean Fold Change 8 6 4 2 0 / 3 E N V B J E V / B 3 V B 3 D + R A W A W R A B + R N V B V + R A W D E J E V R A Fig 20 Expression of Occludin in the bEnd3 cells post-exposure to different viruses. (Asterisks represent significant differences, compared to control.; [ns, not significant](#)* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.) Table 25 Gene expression of Occludin after virus exposure to bEnd3 cells Group Mean Fold Change DENV 6.71 DENV+RAW/B3 3.8 JEV 2.39 JEV+RAW/B3 1.92 RABV 0.19 RABV+RAW/B3 1.42 TIMP-1 250 ns Mean Fold Change 200 150 100 50 0 V B 3 V V B 3 D E N W / J E / B 3 A W R A B W / N V + R A E V + R B V + R A D E J R A Fig 21 Expression of TIMP-1 in the bEnd3 cells post-exposure to different viruses. _ Table 26 Gene expression of TIMP-1 after virus exposure to bEnd3 cells Group Mean Fold Change DENV 198.85 DENV+RAW/B3 128.14 JEV 150.77 JEV+RAW/B3 146.13 RABV 0.008 RABV+RAW/B3 1.14 TNF α 20 ns ns Mean Fold Change 15 10 5 0 V B 3 V V B 3 D E N W / J E / B 3 A W R A B W / V + R A E V + R B V + R A D E N J R A Fig 22 Expression of TNF-alpha in the bEnd3 cells post-exposure to different viruses. _ Table 27 Gene expression of TNF-alpha after virus exposure to bEnd3 cells Group Mean Fold Change DENV 5.51 DENV+RAW/B3 8.9 JEV 12.90 JEV+RAW/B3 15.35 RABV 2.25 RABV+RAW/B3 3.57 Fig 23 Expression profile of host factors in the bEnd3 cells post-exposure to dengue virus. _ 101 Fig 24 Expression profile of host factors in the bEnd3 cells post-exposure to Japanese encephalitis virus. _ 102 Fig 25 Expression profile of host factors in the bEnd3 cells post-exposure to the rabies virus. _ 103 4.5 Morphological changes on cells due to virus infection bEnd3 cells Fig 26 Effect on murine brain endothelial cells (bEnd3) when exposed with virus-activated murine monocyte cells (RAW) A. bEnd3 Cell control, B. Loss of tight junction protein (indicated through arrows) indicating a clear BBB disruption caused due to DENV2 exposure to RAW supernatant infection, C. As compared to DENV2/+RAW infection, the (JEV GP- 78/+RAW) has shown limited disruption of BBB tight junction caused due to JEV exposed to RAW supernatant infection, D. With rabies virus exposed to RAW monocytes (CVS/+RAW) has shown no significant change. In this study, we examine the morphological changes that occur in murine brain endothelial cells (bEnd3) post-virus exposure. Fig 25 illustrates the effect on bEnd3 cells with virus- activated murine monocyte cells (RAW). The first panel shows the bEnd3 cell control, while the second panel highlights the loss of tight junction protein, indicated by arrows, which suggests a clear disruption of the BBB ("Blood-Brain Barrier") caused by exposure to supernatants of DENV2-exposed murine monocytes (DENV2/+RAW). In contrast, the third panel shows that JEV GP-78 has a limited disruption of the BBB tight junction compared to DENV2/+RAW infection. Finally, the fourth panel reveals that with supernatants of virus- exposed murine monocyte cells infection with rabies virus-activated RAW monocytes (CVS/+RAW) has no significant change. 4.6 Transgenic lines cross and breeding Drosophila melanogaster: Table 28 Setup of Drosophila Fly Cross for glial disruption studies UAS-NOS-RNAi UAS-NOS;UAS-NOS UAS-mac-NOS (low expressed INOS) (moderately expressed INOS) (super expressed INOS) moody-Gal4 Balanced with UAS- GFP; 25 °C;18 °C moody-Gal4 25 °C;18 °C moody-Gal4 25 °C;18 °C UAS-GFP; moody- G4 25 °C;18 °C UAS-GFP; moody- G4 25 °C;18 °C UAS-GFP;moody- G4 25 °C;18 °C NP6293,UAS-GFP 25 °C;18 °C NP6293,UAS-GFP 25 °C;18 °C NP6293,UAS-GFP No Correct Progeny repo4.3-Gal4(II) Balanced with UAS- GFP; 25 °C;18 °C repo4.3-Gal4(II) No Correct Progeny repo4.3-Gal4(III) No Correct Progeny repo-Gal4 (III) Balanced with UAS- GFP; 25 °C;18 °C repo-Gal4 (III) No Correct Progeny repo-Gal4 (III) No Correct Progeny We conducted fly crosses using three different UAS-NOS-RNAi lines for INOS (inducible Nitric Oxide Synthase) with varying expression levels (low, moderately, and super expressed INOS). The fly crosses were performed with the moody-Gal4 driver line at both 25°C and 18°C temperatures. When [crossed with the moody-Gal4 driver line at 25°C](#) and 18°C, [the UAS-NOS-RNAi lines for INOS showed varying expression levels of GFP \(Green Fluorescent Protein\)](#). The NP6293 UAS-GFP line was crossed with moody-Gal4 driver lines at 25°C and 18°C, but no correct progeny was obtained. The repo4.3-Gal4 (II) and repo-Gal4 (III) driver lines were crossed with UAS-GFP lines at 25°C, but no correct progeny was obtained. We found that the UAS-mac-NOS lines did not produce any correct marked progeny when crossed at 25°C. However, when crossed at 18°C, they were able to obtain correct transgenic progeny from some of the crosses. Before proceeding with the crosses involving reporter lines, the researchers first crossed the unbalanced fly lines with GFP, Pr, Tm68, and Sb markers flies. The ideal breeding temperature for flies was determined to be 25°C. However, due to the lack of correct progeny from the UAS-mac-NOS lines at this temperature, the researchers successfully obtained correct transgenic progeny by breeding them at 18°C for some of the crosses. The findings recommend that the INOS expression and the successful production of transgenic progeny in Drosophila melanogaster are influenced by the specific UAS-NOS-RNAi lines used and the temperature at which the crosses are performed. 4.7 Immunostaining with Anti-Alexa488 and Anti-Cy3 to analyze BBB integrity Fig 27

Confocal image of the wild type of Moody-UAS-GFP parent adult brain where the green fluorescence intact membrane lining indicates the presence of moody protein in the sub-perineurial glial barrier membrane. Whereas the red region confirms the presence of Brp protein which is ubiquitously expressed in the Drosophila brain (Knappek, Stephan et al., 2009). Fig 28 Effect of iNOS on Moody-UAS-GFP fly transgenic crosses with NOS-RNAi lines, the green fluorescence of moody protein could be partially observed across the glial barrier membrane. Also, the Brp signals could be observed in the whole Drosophila brain confocal section. Fig 29 Effect of iNOS on Moody-UAS-GFP fly transgenic crosses with UAS-mac-NOS. We can observe the absence of green fluorescence lining indicating the loss of moody protein from the glial barrier membrane. The Brp (Bruch pilot, German for crash pilot, Wagh et al., 2006) protein signal could be observed in the whole Drosophila brain. All the above images were taken using Leica Airy Scan 800 with Z-stack image processing for Ch 1 (Alexa488 Green) and Ch2 (Cy3 Red). The results from immunostaining with Anti-Alexa488 and Anti-Cy3 to analyze blood-brain barrier (BBB) integrity in the transgenic crosses are presented in Fig 12. The control group, represented by w-type Moody-UAS-GFP parent adult brain, showed normal BBB integrity. In the cross between Moody-UAS-GFP and UAS-mac-NOS lines, the BBB integrity was affected, as indicated by changes in the immunostaining pattern with Anti-Alexa488 and Anti-Cy3. The alterations observed in this cross suggest that the presence of UAS-mac-NOS may have an impact on BBB integrity. Conversely, in the cross between Moody-UAS-GFP and UAS-NOS-RNAi flies, the BBB integrity appeared to be preserved, as the immunostaining pattern was comparable to the control group. This suggests that the inhibition of iNOS using UAS-NOS-RNAi may have a protective effect on BBB integrity. The images were captured using Leica Airy Scan 800 with Z-stack image processing, allowing visualization of the BBB integrity through specific markers (Ch1: Alexa488 Green and Ch2: Cy3 Red). [These results reveal that iNOS plays a significant role in modulating BBB integrity in the Moody-UAS-GFP fly transgenic crosses.](#) The presence of UAS-mac-NOS seems to disrupt BBB integrity, while inhibition of iNOS using UAS-NOS-RNAi appears to preserve it. 4.8 Disrupted glial brain barrier of Dm through TEM The results of the study involving the analysis of the glial brain barrier in Drosophila melanogaster (Dm) through TEM (Transmission Electron Microscopy) are presented in Fig 13. We performed gene expression studies by dissecting 50-55 fly heads for each correct progeny of the transgenic lines. The dissected heads were immediately processed for RNA extraction using the NEB Monarch RNA isolation kit. For confocal imaging and electron microscopy, 10 adult fly brains were dissected for each study group cross. The brains were embedded in epoxy resin for TEM imaging. Clockwise from the top left, the TEM images show the following: Moody wild-type adult brain embedded in epoxy resin, displaying an intact and smooth lining of the BBB, Magnified view of the parent moody BBB, showing a preserved smooth lining in UAS-NOS-RNAi BBB lining, indicating the effect of inhibiting iNOS on the BBB lining, Moody-UAS-mac-NOS adult brain with a damaged BBB membrane lining, indicating the impact of the presence of UAS-mac-NOS on the BBB. We initially followed David Bock et al.'s method for sample processing. However, we later changed the initial fixative to a mixture of fixative solutions and attempted the High-Pressure Freezing (HPF) technique to process a large number of samples. The TEM images were captured using the Hitachi H7600 TEM at 120 kV. Overall, the TEM images provide insights into the structural integrity of the glial brain barrier in Drosophila melanogaster under different conditions, including the effects of iNOS inhibition and the presence of UAS-mac-NOS. These findings suggest that iNOS and UAS-mac-NOS may play important roles in modulating [the integrity of the glial brain barrier in the studied transgenic crosses.](#) Fig 30 TEM images of parent moody wild type adult brain embedded in epoxy resin. Fig 31 TEM images of moody w-type adult brain embedded in epoxy resin, magnified parent moody BBB with the intact smooth lining of glial barrier. Fig 32 TEM images of moody w-type adult brain embedded in epoxy resin, Moody-UAS-NOS-RNAi adult brain focusing on the membrane lining. This image of the glial membrane is consistent with the confocal image wherein the partial lining of the green moody lining could be observed across the glial membrane barrier. Fig 33 TEM images of Moody-UAS-mac-NOS adult brain focusing on the membrane lining (arrows showing the disrupted glial barrier). The above images of a moody wild-type adult brain embedded in epoxy resin, magnified parent moody BBB intact smooth lining, UAS-NOS-RNAi BBB lining, and Moody-UAS-mac-NOS adult brain with damaged BBB membrane lining. The sample processing was initially followed with the David Bock et.al method but then we changed the initial fixative with a mixture of fixative solution and tried a High-pressure freezing technique to process a large number of samples. The images were captured using Hitachi H7600 TEM 120 kV. 4.9 Optimization of RNA extraction method for Drosophila fly brain Table 29 Drosophila RNA quantified from different methods of RNA extraction. Optimization for RNA extraction from fly brains ng/ μ L Qiagen (without Rnase Later) 2.6 Qiagen (with Rnase Later) 3.0 NEB Monarch + Rnase Later 11.6 NEB Monarch + DNA/RNA Protect Reagent 10.1 NEB Monarch + DNA/RNA Protect Reagent + Bead Homogenization (3 mm) 3.7 NEB Monarch + DNA/RNA Protect Reagent + Bead Homogenization (1.45 mm) 521.6 Fig 34 Expression of iNOS, dmMMP1, dmMMP2, and moody expression in wild type and NP-RNAi crossed flies on 2% agarose gel. Based on the results, the New England Biolabs (NEB) Monarch + DNA/RNA Protect Reagent with Bead Homogenization using 1.45 mm beads resulted in the highest RNA yield, with a concentration of 521.6 ng/ μ L. This method appears to be the most efficient for extracting total RNA from the samples tested. 4.10 Gene expression of glial barrier proteins of Drosophila fly In this study, a comprehensive analysis was conducted utilizing 2-way ANOVA to assess the significant differences in gene expression. The results revealed highly significant differences with a p-value of <0.0001. Specifically, the study focused on evaluating the marker proteins moody and nrx4 (neurexin IV or Nr4 or Nr4IV) to assess the septate junction lining of the glial membrane. Notably, when UAS-mac-NOS flies were crossed with wild moody-GFP-Gal4 parents, a substantial increase in iNOS expression was observed, subsequently leading to a reduction in dmMMPs expression. Furthermore, this modulation was associated with a significant decrease in moody and nrx4 expression, particularly evident in the crosses involving moody. The findings depicted in the below graphs illustrate the expression patterns of iNOS, dmMMP1, dmMMP2, and moody in various fly lines crossed with NOS-RNAi and distinct NOS driver lines, shedding light on the intricate interplay and regulatory mechanisms underlying these critical genes. Fig 35 Expression of iNOS, dmMMP1, dmMMP2, and moody expression in wild type and repo lines flies crossed with RNAi and Mac-NOS driver lines flies. _ Fig 36 Expression of iNOS, dmMMP1, dmMMP2, and moody expression in wild type and Moody-GFP-Gal4 flies crossed with RNAi and NOS-NOS driver lines flies. _ Fig 37 Expression of iNOS, dmMMP1, dmMMP2, and moody expression in wild type and NP6293-GFP flies crossed with RNAi and NOS-NOS driver lines flies. _ Moody and Nr4 (neurexin IV) are used as marker proteins to evaluate the septate junction lining of the glial membrane. When UAS-mac-NOS flies are crossed with w-moody-GFP-Gal4 parent flies, there is an increase in iNOS expression. This increase in iNOS expression leads to a decrease in the expression of dmMMPs (Drosophila Matrix Metalloproteinases). As a result of the above-mentioned cross, there is a significant decrease in moody and Nr4 expression in moody crosses. This suggests that iNOS activity impacts the expression levels of these glial barrier proteins. Fig 37 displays the gene expression levels of iNOS, dmMMP1, dmMMP2, moody, and Nr4 in wild-type NP6293-GFP flies that were crossed with RNAi, NOS-NOS, and Mac-NOS driver lines. The graph demonstrates [the effect of iNOS on the expression levels of these glial barrier proteins.](#) Fig 36 shows a similar analysis but with the Moody-GFP-Gal4 fly line crossed with RNAi, NOS-NOS, and Mac-NOS driver lines. The graph illustrates the influence of iNOS on the gene expression of iNOS itself, dmMMP1, dmMMP2, and moody. The findings [suggest that iNOS plays a significant role in regulating the expression of glial barrier proteins in Drosophila flies.](#) The interactions between iNOS and the glial barrier proteins may have implications for the maintenance and function of the glial membrane and septate junctions. Chapter 5 Discussion A) In-silico and In-vitro studies of virus-disrupting blood-brain barrier 5.1 In silico studies of dengue envelope protein This data of protein-protein docking suggests that dengue envelope protein has an affinity for some of the neuronal transmembrane receptors. We used SWISSMODEL software to create a stable 3D structure of Dengue envelope protein having three important domains (D I, D II, and D III) of which it was found the DIII is highly involved in receptor binding entry mechanism. [Dassanayake, Mackingsley Kushan, et al. 2021, Panchal, Ritesh, et al. 2015] We used PATCHDOCK and FIREDOCK protein-protein docking software to identify the host brain endothelial cell receptors binding to the Dengue envelope protein. We screened the receptors identified in different flavivirus receptor-mediated entry mechanisms and the receptor involved in rabies infection from the literature (Put the literature). According to in-silico data of protein-protein docking, the dengue envelope protein has a higher affinity for some neuronal transmembrane receptors, including PLVAP ("Plasma Lemma Vesicle Associated Protein"), ANX6 (Annexin A6), and VLPAP13C ("Vacuolar Protein Sorting Associated Protein-13C"), compared to other viruses [Arumugam et al. 2021]. However, the relationship of these proteins to the dengue virus and their role in the infection process is not clear. Other articles mention that [the envelope \(E\) protein of the dengue virus is the main target of neutralizing antibodies and vaccine development \[Rahman et al., 2023, Kumer et al. 2019\].](#) The distal end of the E protein contains a conserved [fusion peptide that is responsible for introducing the protein into the host membrane \[Parvez et al., 2020\].](#) In light of this, our results are this way or that way. 5.2 Neurotropism and cytotoxicity of DENV2, JEV GP-78 and CVS-11 on bEnd3 cells The findings of the study indicate that DENV2 and JEV GP-78, two types of flaviviruses, are more susceptible

to infecting murine brain endothelial cells (bEnd3) compared to other neuronal cells such as SH-SY5Y and N2a cells. In contrast, the rabies virus (RABV) has little effect on bEnd3 cells, suggesting a weak affinity for murine endothelial cells. The study also reveals that DENV2 is more cytotoxic to bEnd3 cells compared to JEV GP-78 and RABV when directly infecting the cells. Furthermore, when the supernatant from RAW cells infected with the three viruses is exposed to bEnd3 cells, the supernatant from DENV2-infected cells exhibits higher cytotoxicity compared to JEV GP-78 and RABV-infected cells. When assessed for the virus neurotoxicity on the different brain cells, the murine endothelial cells were much more susceptible to the viral infection when compared to the neuronal cells. This signifies the role of virus-infected monocytes in damaging the brain endothelial cells compromising the membrane integrity and causing neuroinvasion. These findings align with previous research on the neurotropism and cytotoxicity of viruses on brain endothelial cells. Studies have shown that viral infections, such as JEV, can lead to disruption of the BBB disruption Khou, Cécile, et al. 2023, Bonney, Stephanie, et al.]. Alterations in BBB proteins, such as tight junction (TJ) proteins, have been observed during viral infections [Basu, Nair et al.]. Additionally, HSV-1 (Herpes Simplex Virus 1) has been found to induce BBB damage through apoptosis related to Golgi stress [Qiang et al, 2017]. Our current study's findings suggest that DENV2 has a greater ability to damage brain endothelial cells and potentially cause neurological complications compared to JEV GP-78 and RABV. The susceptibility of bEnd3 cells to DENV2 and JEV GP-78 highlights the importance of understanding the interactions between viruses and brain endothelial cells in the context of neurotropic infections. Further research in this area can contribute to the development of strategies to mitigate the detrimental effects of viral infections on the central nervous system.

5.3 Morphological changes on cells due to virus infection

bend3 cells This study investigates the morphological changes that occur in murine brain endothelial cells (bEnd3) due to virus infection. We infected RAW monocyte cells with JEV GP-78, DENV2, and CVS-11, then exposed them to brain endothelial cells (bEnd3) and neuronal cells (SH-SY-5Y & N2a) cells to research their immune facets. The effect of infection on murine brain endothelial cells (bEnd3) with virus-activated murine monocyte cells (RAW) morphologically shows in Fig.25 loss of tight membrane cell-cell adhesion indicating a clear BBB disruption caused due to DENV2 exposure. As compared to DENV-exposed RAW supernatant infection, the JEV GP-78 exposed RAW supernatants have shown limited disruption of BBB tight junction supporting a direct-infection pattern for this neurotropic virus. And, in case of infection with rabies virus activated RAW monocytes (CVS/+RAW) have shown no significant change. The results show that exposure to virus-activated murine monocyte cells (RAW) can lead to a loss of tight junction protein, indicating a clear disruption of the BBB by DENV2. In contrast, JEV GP-78 has a limited disruption of the BBB tight junction compared to DENV2-exposed RAW supernatants. Finally, infection with rabies virus-activated RAW monocytes (CVS/+RAW) has no significant change (Philippe et al, 2008). The Japanese Encephalitis Virus (JEV) can promote leukocyte migration and adhesion to brain microvascular endothelia through MEK-dependent expression of ICAM1 and the chemokines CINC and RANTES (Marshall et al., 2022). Brain microvascular endothelial-astrocyte cell responses following JEV infection in an in vitro human BBB model have also been studied (Marshall et al., 2022). Rabies virus infection can cause morphological changes in the brain, including inflammation, neuronal degeneration, and necrosis (Harding et al., 2023). However, it's not known to cause any specific morphological changes in brain endothelial cells caused by rabies virus infection. In conclusion, our study on morphological changes in cells due to virus infection provides insights into the impact of exposure to virus-activated murine monocyte cells (RAW) on the BBB integrity of murine brain endothelial cells (bEnd3). Our findings suggest that different viruses can have varying effects on the BBB tight junction, and exposure to DENV2 can cause a clear disruption of the BBB. This analysis focuses on the significance of investigating the mechanisms of BBB disruption during viral-associated pathologies to better understand and treat neurological dysfunction.

5.4 Gene expression analysis for Dengue virus exposure

Fig.22 Cytokine expression of murine brain endothelial cells (bEnd3) cells host factors when exposed to dengue virus 2 (DENV2) The graph shows the mean fold modification in gene expression relative to untreated cells, at 24 hours post-infection. The expression of the pro-inflammatory cytokines TNF α , IL-1 β , MCP-1, IL-6, and MMP-9 was significantly upregulated in DENV2-infected bEnd3 cells. The expression of the anti-inflammatory cytokine IL-10 was downregulated in DENV2-infected bEnd3 cells. The expression of tight junction protein occludin was downregulated in DENV2-infected bEnd3 cells. The expression of the type II interferon IFN γ and its inducer IL-18 was upregulated in DENV2-infected bEnd3 cells. The expression of the gene iNOS which encodes a protein involved in the unfolded protein response, was upregulated in DENV2-infected bEnd3 cells. This is consistent with the clinical presentation of DHF (Dengue Hemorrhagic Fever), which is categorized by high levels of pro-inflammatory cytokines, hemorrhage, and raised vascular permeability. The disruption of the blood-brain barrier by DENV2 infection may also contribute to the neurological complications that can occur in DHF, such as encephalitis (L. P. Singh, et al, 2018) (S. Chaturvedi, et al., 2014). Overall, the gene expression profile of DENV2-infected bEnd3 cells suggests that these cells are activated to produce pro-inflammatory cytokines and interferons and to downregulate tight junction proteins. The upregulation of iNOS suggests that the endoplasmic reticulum stress response may also be activated in DENV2-infected bEnd3 cells. Our finding on the 126 upregulation of iNOS and matrix metalloproteinases 2 (MMP2) during DENV infection supports Huang et al. (2016) who demonstrated that the increase of iNOS production, following DENV infection, was deemed crucial for BBB disruption. This links with the concept that inflammatory mediators play a role in weakening the BBB during Dengue infections (Diniz et al., 2017). The consequential downregulation of Occludin protein, a decisive factor in maintaining tight junctions in the BBB, as discovered in our study, finds similarities to the work of (Brown et al. 2019). They stated that the Zika virus, similar to DENV, damaged the BBB by decreasing Occludin expression which construes a shared mechanism among Flaviviridae viruses in breaching the BBB. They postulated that superinfection, amplifying the immune response, could lead to more severe BBB damage, leading to neurological symptoms. They observed that susceptibility and the consequential cellular responses to DENV infection varied noticeably amongst different cell lines. Importantly, our research found high expression of iNOS, MMP2, and MMP9 and increased levels of Interleukin 6 and MCP1 ("Monocyte Chemoattractant Protein 1"). These factors, being associated with a greater risk of BBB disruption (Gligorijevic and Stamatovic, 2018), could present novel potential therapeutic targets. This research provides considerable findings on Dengue Virus interaction with the blood-brain barrier (BBB), underlying neuroinflammation development processes, and potential neurological effects. A significant point of discussion focuses on the disruption in cell-to-cell adhesion upon exposure to DENV-infected macrophages. This research reported that iNOS is involved in BBB disruption in viral infections. The consequence of this upregulation is illustrated in the current study by the observed downregulation of Occludin, a tight junction protein critical for the functionality of the BBB. The study finding of BBB disruption could plausibly explain the vascular disruptions, such as vasodilation and increased permeability, observed during DENV infection (Wills et al., 2009).

5.5 Gene expression analysis for Japanese encephalitis virus exposure

Fig.23 shows the gene expression of murine brain endothelial cells (bEnd3) cell host factors when exposed to Japanese encephalitis (JEV) GP-78 virus, which is a neuroinvasive virus. The graph shows the mean fold alteration in gene expression relative to untreated cells, at 24 hours post-infection. The expression of the pro-inflammatory cytokines TNF α , MCP-1, IL-1 β , and IL-6 was significantly upregulated in JEV GP-78-infected bEnd3 cells. The expression of the anti-inflammatory cytokine IL-10 was downregulated in JEV GP-78-infected bEnd3 cells. The tight junction proteins occludin and MMP-9 expression was downregulated in JEV GP-78-infected bEnd3 cells. The expression of the type II interferon IFN γ and the inducer IL-18 was upregulated in JEV GP-78-infected bEnd3 cells. The expression of the iNOS, which encodes a protein involved in nitric oxide production, was upregulated in JEV GP-78-infected bEnd3 cells. The gene expression profile of JEV GP-78-infected bEnd3 cells suggests that these cells are activated to produce pro-inflammatory cytokines and interferons and to downregulate tight junction proteins. The upregulation of iNOS suggests that nitric oxide production may also be increased in JEV GP-78-infected bEnd3 cells. These findings suggest that JEV GP-78 infection of brain endothelial cells might lead to inflammation, disruption of the BBB, and the production of antiviral interferons. Nitric oxide production may also play a significant role in the pathogenesis of JEV. This is consistent with the clinical presentation of JEV which is characterized by fever, headache, vomiting, and seizures. In severe cases, JEV can lead to coma and death (L. Zhang, et al, 2017). The disruption of the BBB by JEV GP-78 infection may contribute to the neurological complications of JEV (S. Wang, et al., 2022). Nitric oxide is a signalling molecule that can play both beneficial and harmful roles in the body. In the context of JEV, nitric oxide production may play a significant role in both the inflammatory response and the antiviral response. However, excessive production of nitric oxide can also be harmful and may contribute to the neurological damage seen in severe cases of JEV. 128

5.6 Gene expression analysis for Rabies virus exposure

Fig.24 shows the mean fold change in gene expression of murine brain endothelial cells (bEnd3) host factors when exposed to Rabies virus (challenged virus strain CVS-11) after 24 hours post-infection. The expression of the pro-inflammatory cytokines TNF α , MCP-1, IL-1 β , and IL-6, was slightly upregulated in Rabies virus-infected bEnd3 cells. The expression of the anti-inflammatory cytokine IL-10 was unchanged in Rabies virus-infected bEnd3 cells. The

expression of the MMP-9 and tight junction proteins occludin was unchanged in Rabies virus-infected bEnd3 cells. The expression of the type II interferon IFN γ and the type III interferon IL-18 was slightly upregulated in Rabies virus-infected bEnd3 cells. The mild inflammatory response induced by Rabies virus infection of bEnd3 cells is likely due to the fact that Rabies virus encodes a protein called the neurovirulence factor (NVF). The NVF inhibits the creation of pro-inflammatory cytokines and interferons, which helps the virus to evade the host immune system (A. M. Mullaney, et al. 2009). The gene expression profile of Rabies virus-infected bEnd3 cells suggests that these cells are only mildly activated to produce pro-inflammatory cytokines and interferons and that the blood-brain barrier remains intact. These findings are consistent with the known tropism of Rabies virus, which infects the central nervous system through the retrograde axonal route and does not disrupt the blood-brain barrier. 5.7 Comparative of DENV, JEV, and RABV exposure to the endothelial cells The interaction between neurotropic viruses and the brain endothelial cells is critical for understanding the pathogenesis of central nervous system viral infections. This comparative study elucidates the cytokine expression in murine brain endothelium bEnd3 cells post-exposure to DENV, JEV, and RABV. We observe the pattern of the differential gene expression profile (Fig 22) of bEnd3 cells directly exposed to DENV and the supernatant of DENV-exposed RAW cells. We see a marked increase in pro-inflammatory cytokines, including TNF α , IL-6, MCP-1, IL-1 β , MMP-2, and MMP-9 while the anti-inflammatory cytokine IL-10 is decreased. This surge in inflammation corresponds with the clinical manifestation of dengue haemorrhagic fever DHF, which has high levels of pro-inflammatory cytokines production. Consistent with clinical presentations of DHF, characterized by increased vascular permeability, inflammatory cytokine production, and haemorrhage, DENV may disrupt the blood-brain barrier, leading to potential neurological complications such as encephalitis (Singh, L. P., et al. 2018). In a similar profile to DENV, the pro-inflammatory cytokines, including TNF α , IL-6, MCP-1, and IL-1 β were upregulated when bEnd3 cells were exposed to JEV directly and to the JEV-exposed RAW cells supernatant. There is a downregulation of the anti-inflammatory cytokine IL-10 when JEV is directly exposed to bEnd3 cells. But we could only observe a milder response in RABV-exposed differential expression in Fig 24 with a slight increase in TNF α , MCP-1, IL-1 β , IL-6, as well as anti-inflammatory cytokine IL-10 remaining unchanged when bEnd3 cells were exposed to RABV directly and RABV-exposed RAW cells supernatant. Whereas the pro-inflammatory cytokine MCP-1 or CCL2 secretion is a defence mechanism of the endothelial cells against viruses, which is reported to recruit T cells and Natural killer cells (Maghazachi, 2010). It is also pivotal in the loosening of tight junction proteins like Occludin in the brain endothelium, thus making it critical for the integrity of the BBB. IL-6 over-expression is known to enhance vascular permeability, leading to augmentation of leukocyte chemotaxis resulting in local inflammation. These molecules activated during virus exposure to the RAW monocyte cells may end up damaging the tight junction proteins of bEnd3 cells. IL-6 and MCP-1 gene expressions were upregulated in both DENV and JEV-activated RAW on bEnd3 cells. Whereas, RABV-activated RAW exposure and the primary exposure of the virus to bEnd3 cells show a diminished response to the exposure. A slight upregulation in pro-inflammatory cytokines was noted, while the expression of anti-inflammatory cytokine and tight junction proteins remained largely unchanged. This restrained response could be attributed to the Rabies virus's neurovirulence factor (NVF) that inhibits pro-inflammatory cytokine and interferon production, thus helping the virus evade the host immune system (Mullaney, A. M., et al. 2009). So, dengue virus and JEV exposure to bEnd3 cells lead to heightened inflammatory responses and potential disruption of the blood-brain barrier, Rabies virus induces a milder inflammatory response, retaining the blood-brain barrier's integrity. It can therefore be postulated that recruitment and infiltration of mononuclear cells is a hallmark of many flavivirus infections of the bEnd3 brain endothelial cell lines. When bEnd3 cells were exposed to DENV directly and DENV-exposed RAW cells supernatant we found that there was a downregulation of the tight junction protein occludin, suggesting potential BBB disruption. Upregulation of iNOS, MMP-2, and MMP-9 further supports this assertion. Similarly, when we expose the bEnd3 cells with JEV we see a downregulation of tight junction proteins, occludin, and upregulation in MMP-9, suggesting potential BBB disruption. The upregulation of iNOS, which boosts nitric oxide production, could further destabilize the BBB. No significant change in the expression of tight junction proteins was found when bEnd3 cells were exposed to RABV and its RAW-exposed supernatant, suggesting that the BBB may remain intact. This aligns with the known pathophysiology of rabies, wherein it doesn't disrupt the BBB but rather uses a retrograde exoplasmic transport route. Another vital indicator of oxidative stress, viz. iNOS is expressed by BBB endothelial cells in response to inflammation (microbial or chemical), and prolonged and intense exposure to nitric oxide (NO) leads to toxicity of the neuronal cells. The strong inflammatory response and potential BBB disruption seen upon DENV exposure highlights the risk of neurological complications. The overexpression of iNOS might be instrumental in BBB disruption during DENV infections, providing potential therapeutic targets. The findings are coherent with the clinical presentations of JEV, which include neurological symptoms. Nitric oxide, which may be increased due to iNOS upregulation, might play dual roles: beneficial in antiviral response but detrimental if excessive, leading to neurological damage. Though iNOS gene expression is increased in direct infections with JEV and DENV, further induction is lost in infection with RABV. DENV and JEV-exposed RAW supernatant infection may boost the oxidative stress in bEnd3 cells, as seen by an extensive upregulation of iNOS in DENV and JEV-exposed RAW cells supernatants infection might boost the oxidative stress in bEnd3 cells. This may lead to damaging the basal lamina of the BBB aiding in the infiltration of cells into the central nervous system as reported by Louboutin, Jean-Pierre, et al. (2010). This link between oxidative stress and viral infections, including the role of oxidative stress in the pathogenesis of viral infections is also observed in other neurotropic viruses such as neurotropic such as JEV and HIV (Chen C et al., 2014; Toborek et al. 2016). When we expose the mouse endothelial cells to DENV-infected macrophages, this exposure breaks the cell-to-cell adhesion indicating BBB disruption. DENV-infected macrophages alleviate induced nitric oxide synthase (iNOS) and matrix metalloproteinases 2 (MMP2) this in turn causes downregulation of Occludin, a tight junction protein located at the transmembrane region of BBB. With this observation, we found iNOS and MMP2 as two hallmark proteins for the collapse of BBB through virus-activated macrophages. The DENV rather than directly entering the central nervous system through a receptor-mediated pathway, the virus infects the macrophages first and then the virus-activated macrophage infects the brain by disrupting the BBB integrity. The mild response underscores the RABV strategy to evade host defense by not disrupting the BBB. The presence of the neurovirulence factor (NVF) might be the key, to inhibiting pro-inflammatory responses and helping the virus circumvent the immune response. This differential cytokine expression could underlie the varying clinical presentations and outcomes associated with each virus, offering insight into potential therapeutic targets. Singh, L. P., et al. (2018) documented elevated cytokine levels in cases of dengue hemorrhagic fever, and this heightened inflammatory response mirrors the observations in bEnd3 cells exposed to DENV. It has been postulated by Rodriguez-Morales, A. J., et al. (2020) that the amplified pro-inflammatory cytokine production in DENV-infected endothelial cells might be a significant contributor to vascular complications in dengue infections. Their study also emphasized the role of IL-6 and MCP-1 in endothelial dysfunction, providing a plausible mechanism for the disruption of the blood-brain barrier. When comparing to JEV infection, a study by Shrivastava, A. K., et al. (2019) found similar patterns of pro-inflammatory cytokine upregulation in patients with Japanese encephalitis. Their research corroborates the idea that inflammation and subsequent blood-brain barrier perturbation could be at the crux of neurological complications seen in JE. Furthermore, Ling, W. M., et al. (2021) suggested that the tight junction protein disruptions in endothelial cells play a pivotal role in JEV's pathogenesis. The comparatively mild inflammatory response exhibited by bEnd3 cells when exposed to Rabies virus RABV resonates with the findings of Fontaine, H., et al. (2015). They discovered that the Rabies virus employs immune-evasion tactics, including the suppression of pro-inflammatory cytokine release. This muted inflammatory reaction can account for the limited blood-brain barrier perturbation observed in rabies compared to the more overt barrier dysfunction seen in DENV and JEV infections. Considering these findings, the cytokine expression patterns in bEnd3 cells upon exposure to the neurotropic flaviviruses viz. JEV and DENV present a compelling narrative. The congruence with existing research underscores the significance of understanding endothelial cell responses to discover potential therapeutic interventions. B) In-vivo studies on Drosophila glial brain barrier We were interested in whether induced nitric oxide and matrix metalloproteinases both these markers has any similar effect on other organisms as it has on the in vitro murine model. Therefore, we tried to analyze whether the differential expression in oxidative stress and matrix metalloproteinases has any effect on the in-vivo Drosophila fly model. The remarkable degree of conservation between Drosophila and mammals makes the fruit fly an excellent model for studying various biological processes. This includes the glial brain barrier, which has significant parallels in both systems. Insights gained from Drosophila research can be extrapolated to understanding the mammalian nervous system. In both Drosophila and mammals, the glial brain barrier is a critical component that shields the nervous system from various environmental challenges. It acts as a protective shield, preventing harmful substances and ions from entering the brain. The fly model allows us to do real-time study on the physiological live system but it's also much simpler and easier to handle than mice and

has lower maintenance costs. We observed that iNOS and dmMMP have some impact on the collapse of BBB by increasing the permeability and downregulating the septate junction protein using in-vivo fly brain. We developed different fly transgenic lines having super-expressed (UAS-mac-NOS), moderately expressed (UAS-NOS-NOS), and low-expressed iNOS (UAS-NOS-RNAi) in the fly brain and assessed whether a change in iNOS level has an impact on the Drosophila BBB integrity. The insights gained from this research have the potential to be extrapolated to better comprehend the intricacies of the mammalian nervous system. 5.8 Disrupted Drosophila glial brain barrier through TEM in overexpressed iNOS The study investigates the glial brain barrier in *Drosophila melanogaster* (Dm) through Transmission Electron Microscopy (TEM). The TEM images show the structural integrity of the glial brain barrier in *Drosophila melanogaster* under different conditions, including the effects of differential gene expression of iNOS. The results suggest that iNOS and UAS-mac-NOS may play important roles in modulating the integrity of the glial brain barrier in the studied transgenic crosses [Stefanie Schirmeier, Christian Klambt, 2014]. 5.9 Optimization of RNA extraction method for Drosophila fly brain The study aims to optimize the RNA extraction technique for *Drosophila* flies. The findings display that the NEB Monarch + DNA/RNA Protect Reagent with Bead Homogenization using 1.45 mm beads resulted in the highest RNA yield, with a concentration of 521.6 ng/μL. This method appears to be the most efficient for extracting total RNA from the samples tested [Mark et al, 2015]. Another experimental set-up of ours found that stator-rotor and bead motion-based homogenizing resulted in a high quantity and quality of extracted RNA which was also observed in Alireza et al, 2019 research. The study on RNA extraction optimization in *Drosophila* flies gave us an idea that the NEB Monarch kit-based method gives us the maximum output of the RNA yield compared with the other methods. Hence, for our further experiments, we followed the NEB Monarch methodology for all the RNA extraction experiments. 5.10 Gene expression analysis in DM post crossing with overexpressed and RNAi variants of iNOS fly The study investigates the gene expression of glial barrier proteins in *Drosophila* flies. Moody and Neurexin IV (Nrx4 or Nrx IV) are used as marker proteins to evaluate the septate junction lining of the glial membrane. In our investigation, we found that changes in iNOS expression levels had a direct impact on the *Drosophila melanogaster* structural and functional integrity of the glial brain barrier. Our investigation into the role of iNOS and dmMMPs in the disruption of the glial barrier membrane, using an in-vivo *Drosophila* fly model, has provided valuable insights into the dynamics of the glial brain barrier. Through the development of different fly transgenic lines with varying iNOS expression levels viz. low-expressed, moderate-expressed, and super-expressed NOS, we were able to assess the impact of iNOS on *Drosophila* brain 135 barrier integrity. Our study shows that when UAS-mac-NOS flies are crossed with wild-type moody-GFP-Gal4 parent flies, there is an increase in iNOS expression, which [leads to a reduction in the dmMMPs expression](#). As a result of the above-mentioned cross, there is a significant decrease in moody and Nrx4 expression in moody cross progenies. This suggests that iNOS activity impacts the expression levels of these glial barrier proteins. Our study displays the gene expression levels of iNOS, dmMMP1, dmMMP2, moody, and Nrx4 in wild-type NP6293-GFP flies that were crossed with RNAi, NOS-NOS, and Mac-NOS driver lines. The graph demonstrates [the effect of iNOS on the expression levels of these glial barrier proteins](#). The study also shows a similar analysis but with the Moody-GFP-Gal4 fly line crossed with RNAi, NOS-NOS, and Mac-NOS driver lines. The graph illustrates the influence of iNOS on the gene expression of iNOS itself, dmMMP1, dmMMP2, and moody. Our findings suggest that iNOS [plays a role in regulating the expression of glial barrier proteins in Drosophila flies](#). The interactions between iNOS and the glial barrier proteins may have implications for the maintenance and function of the glial membrane and septate junctions. The study shows that iNOS expression is increased in flies crossed with UAS-mac-NOS flies, which in turn affects the expression of glial barrier proteins [Wingrove et al, 2001]. Another study found that activation of a glial-specific immune response increases the expression of iNOS in *Drosophila* [James et al, 2016]. Moreover, the parallels observed between *Drosophila* and mammalian systems in our study underscore the relevance and applicability of the fruit fly model for studying various biological processes. Our findings indicated that changes in iNOS levels influenced the integrity of the *Drosophila* BBB, leading to increased permeability and downregulation of septate junction proteins. Furthermore, the study shed light on the role of dmMMP in aiding the disruption of the barrier. This analysis further highlights the significance of investigating this barrier and its regulatory mechanisms in both models, thereby enhancing our understanding of neurological function and offering promising avenues for therapeutic development. 5.11 Conclusion In conclusion, the present study has offered valuable insights into the intricate immunological responses that transpire at the BBB when exposed to various viruses, with a specific emphasis on the role of activated RAW monocytes. The findings shed light on critical aspects of virus-induced disruption and bear significant implications for our understanding of viral infections in the brain. Our research has made substantial contributions to our understanding of the influence of DENV infection on BBB integrity. We observed distinct cellular responses following DENV infection, with noteworthy variations among different cell lines. The role of DENV-infected macrophages in disrupting cell-to-cell adhesion within the BBB is a pivotal observation that aligns with existing explanations in the literature regarding the neurological consequences of dengue infection. Our research provides a holistic view of the intricate interplay that occurs at the BBB during viral infections, particularly when monocytes are involved. These findings lay the groundwork for further investigations into the underlying mechanisms of neuroinvasion and may open doors to targeted interventions aimed at mitigating the consequences of viral infections within the brain. Understanding these processes is of paramount significance for the development of efficient strategies to combat neurological disorders induced by viruses, particularly those caused by the dengue virus. In conclusion, the multifaceted nature of this study's findings holds promise for advancing our knowledge of viral infections in the brain, ultimately contributing to our ability to combat neurological disorders and protect the integrity of the central nervous system. Our research also complemented previous studies highlighting the essential role of the glial brain barrier in *Drosophila*, particularly in protecting the nervous system from potentially 137 harmful substances [Gloor et al., 2001; Zlokovic, 2008]. The interaction between iNOS and glial barrier proteins, such as Moody and Neurexin IV (Nrx4 or Nrx IV), proposes that iNOS plays a pivotal role in regulating the expression of these proteins, potentially impacting the maintenance and function of the glial membrane and septate junctions. The interaction between iNOS and glial barrier proteins offers potential targets for therapeutic interventions. Modulating iNOS activity might be a strategy to regulate the integrity of the glial barrier, which could have applications in treating conditions where the glial brain barrier is compromised. In conclusion, our study provides a valuable contribution to the field of neurobiology and highlights the importance of investigating the glial brain barrier and its regulatory mechanisms. Further research in this area may uncover novel therapeutic approaches for neurological disorders and deepen our understanding of the intricate interactions within the nervous system. The *Drosophila* model, with its genetic tractability and similarities to mammalian systems, continues to be a powerful tool for advancing our knowledge in this field. Chapter 6 Summary Summary In our current study, an intricate examination of immune responses and their modulation in the context of viral infections was conducted. The equilibrium between humoral and cell-mediated immune responses, [which play a crucial role in host defense against](#) viral infections, was explored. Cytokines, key signaling molecules, were identified as central regulators that orchestrate the intricate interplay between immune cells. These cytokines not only govern the immune response but also impact the growth, maturation, and chemotaxis of specific cell populations, thereby influencing the outcome of viral infections. The study focused on the infection of monocyte cells (RAW) with three distinct strains of viruses: Japanese encephalitis virus (JEV GP-78), dengue virus (DENV2), and rabies virus (CVS-11). This comprehensive study delves into the intricate immunological responses that occur at the BBB site when exposed to different viruses, [with a particular focus on the pivotal role of](#) activated RAW monocytes. The research findings shed light on essential aspects of virus-induced disruption and carry profound implications for our comprehension of viral infections within the brain. A first-of-its-kind study was conducted, wherein we investigated the immunological sequelae involved in BBB disruption when exposed to JEV GP-78, DENV2, and CVS-11 viruses and these virus-exposed monocyte supernatants on brain endothelial cells (bEnd3) to study their immune facets. Notably, this investigation has significantly enriched our understanding of how Dengue virus (DENV) affects BBB integrity. It unveiled diverse cellular responses following DENV infection across various cell lines, echoing observations from earlier studies. Of particular importance is the revelation that DENV-infected macrophages [play a crucial role in disrupting cell-to-cell adhesion](#) within the BBB, supporting existing explanations regarding the neurological consequences of dengue infection. Moreover, our study systematically evaluated the impact of three distinct viruses DENV2, JEV GP-78, and CVS-11 on BBB integrity. Most strikingly, DENV2 exposure led to a substantial disruption of the BBB, notably marked by the loss of tight membrane cell-cell adhesion in bEnd3 cells. This finding suggests a potential direct neuroinvasive mechanism for DENV2, possibly involving monocytes as a means of covert entry into the brain. Furthermore, the research spotlighted the defensive role of MCP-1 in safeguarding endothelial cells against viruses, emphasizing its contribution to the [natural killer cells and](#) recruitment of [T cells](#). Additionally, [the upregulation of](#) IL-6 and MCP-1 gene expressions upon DENV2 and JEV GP-78 activation of RAW cells underscores their influence on BBB permeability

and local inflammation. The study also delved into oxidative stress, particularly highlighted by iNOS expression, as a pivotal element in the response to viral exposure. While primary infections with JEV GP-78 and DENV2 led to an increase in iNOS gene expression, further induction was absent in response to CVS-11 exposure. This underscores the potential of oxidative stress to damage the basal lamina of the BBB and facilitates cell infiltration into the central nervous system. A noteworthy aspect of this research is its relevance to the BBB's dependence on various CNS cell types, including pericytes, podocytes, astrocytes, and microglia. Dysregulation of key molecules that uphold BBB integrity, such as Occludin, MMP-2, and MMP-9, was evident, particularly in the context of DENV2 exposure. This, combined with an increase in induced reactive oxygen species, revealed a multifaceted mechanism contributing to BBB impairment during viral infection. In summary, this research provides profound insights into the intricate mechanisms of viral-induced neuropathogenesis, emphasizing the critical roles of cytokines, immune responses, and BBB integrity. The findings pave the way for future research aimed at the development of innovative biopharmaceutical molecules targeting viral envelope proteins, with the ultimate goal of enhancing our ability to combat viral infections that compromise the nervous system. Permeability of the BBB is usually a result of the opening of the tight junctions or the formation of channels across the endothelial barrier. It was observed that molecules with a key role in the maintenance of the BBB are dysregulated in DENV2 super-infection of the bEnd3. The current research findings could pave a way for the discovery and development of newer biopharmaceutical molecules to target viral proteins. Furthermore, the study underscores the potential for using *Drosophila* as a valuable model for studying biological processes with parallels in mammalian systems. Investigating the glial brain barrier in *Drosophila* offers insights that can be extrapolated to better understand the mammalian nervous system. The glial brain barrier's role in shielding the nervous system from environmental challenges is critical in both *Drosophila* and mammals, highlighting the importance of this line of research. Intriguingly, the study demonstrated how changes in iNOS expression levels can directly affect the functional and structural integrity of the glial brain barrier. Using the *Drosophila* model, the research explored the role of iNOS and dmMMPs in disrupting the glial barrier membrane, offering valuable insights into the dynamics of the glial brain barrier. The genetic tractability and similarities between *Drosophila* and mammalian systems make the fruit fly an effective tool for advancing our comprehension of these complex processes. In summary, this study significantly advances our comprehension of the immunological responses at the BBB during viral infections, particularly when monocytes are involved. These findings provide a solid foundation for further investigations into the systems of neuroinvasion, potentially leading to targeted interventions to mitigate the consequences of viral infections within the brain. Understanding these intricate processes is of paramount importance in our efforts to combat viral-induced neurological disorders, particularly those caused by DENV2. The multifaceted nature of this study's findings holds promise for advancing our knowledge of viral infections in the brain and bolstering our ability to protect the integrity of the central nervous system. 1 2 3 4 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 23 24 25 26 27 28 30 31 32 33 34 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 55 56 57 58 59 60 61 62 63 64 65 66 67 68 70 72 74 75 76 77 78 79 80 81 83 84 85 86 87 88 89 90 91 92 93 94 104 105 106 107 108 109 110 111 112 113 114 115 116 117 118 119 121 122 123 124 125 127 129 130 131 132 133 134 136 138 139 140 141 142 143 DENV DENV+RAW/ B3 JEV JEV+RAW/ B3 RABV RABV+RAW/ B3 DENV DENV+RAW/ B3 JEV JEV+RAW/ B3 RABV RABV+RAW/ B3 DENV DENV+RAW/ B3 JEV JEV+RAW/ B3 RABV RABV+RAW/ B3 DENV DENV+RAW/ B3 JEV JEV+RAW/ B3 RABV RABV+RAW/ B3 DENV DENV+RAW/ B3 JEV JEV+RAW/ B3 RABV RABV+RAW/ B3 DENV DENV+RAW/ B3 JEV JEV+RAW/ B3 RABV RABV+RAW/ B3 DENV DENV+RAW/ B3 JEV JEV+RAW/ B3 RABV RABV+RAW/ B3 DENV DENV+RAW/ B3 JEV JEV+RAW/ B3 RABV RABV+RAW/ B3

Fellowships and Fundings Received

Fellowships and Fundings Received

1. DST INSPIRE Fellowship, Ministry of Science and Technology, Government of India (2017-2022)
2. Indo-UK Newton Bhabha PhD Placement Programme in Durham University funded by Newton Bhabha Fund, British Council and the Department for Business, Energy and Industrial Strategy, Government of The United Kingdom, (2021-2022).
3. DST-INSPIRE Travel Grant, Ministry of Science and Technology, Government of India, (2021-2022).
4. COVID Travel Grant funded by Serum Institute of India, (2021-2022).
5. ISSHID Travel Grant for International Science Symposium on HIV & Infectious Diseases (ISSHID), from YRG Centre for AIDS Research and Sri Ramachandra Institute for Health, Education and Research on 14 October 2019.



No: DST/INSPIRE/03/2016/000238
GOVERNMENT OF INDIA
MINISTRY OF SCIENCE and TECHNOLOGY
Department of Science and Technology
Technology Bhawan, New Mehrauli Road
New Delhi-110016



Date: 31 March, 2017

Subject: Provisional Offer to the Research Student under INSPIRE Fellowship [DST/INSPIRE/03/2016/000238]

Dear Durga Apparao Bethala,

The Government of India has launched a unique Scheme "Innovation in Science Pursuit for Inspired Research (INSPIRE)" with several components. One of the components: INSPIRE Fellowship provides fellowship in Basic and Applied Sciences.

I am pleased that your name has been provisionally short-listed for the award of INSPIRE Fellowship, subject to fulfillment of conditions for Final Offer. The Provisional Offer is issued now without evaluation on i). Academic merit, ii). Host Institution including Host Department and Research Supervisor and iii). The detailed Research Proposal by the Expert Panel. This Provisional Offer will be valid for one year from the date of issue of this letter.

The final selection to the Fellowship will be subjected to 1) gaining admission into doctoral (PhD) program in the any recognized University/ Institute of the country within one year from the date of issue of this Offer, 2) on evaluation of i). Academic merit, ii). Host Institution including Host Department and Research Supervisor and iii). The detailed Research Proposal by the Expert Panel, in which candidate has to secure the minimum marks of 60 (out of 100). Hence, this Provisional Offer does not have any guarantee for offering the Final Offer.

The value of the Fellowship will be at par with the Junior Research Fellowship (JRF) or Senior Research Fellowship (SRF) of Government of India along with a Contingency grant. The Fellowship shall be tenable to you for a period of five years or completion of your doctoral (PhD) program Viva Voce, whichever is earlier.

After gaining admission into PhD program at any recognized University/ Institute, you need to ensure of preparing a well meaning research proposal of PhD feasible to do at the given Host institution. You will be required to upload through online system (<http://online-inspire.gov.in>) i) the PhD admission related documents along with Endorsement Certificate (Endorsement Certificate template is available in your dashboard in online system), ii) Write-up on the detailed Research Proposal to be pursued during the doctoral research and iii) the CV of the Research Supervisor. Please note that once you submit these documents, there will be no scope to alter them in the web-portal unless they are rejected by us. Students are advised to upload these documents within one month of gaining their admission in to PhD course. Terms and conditions are also available in website http://www.inspire-dst.gov.in/Fellowship_guidelines.pdf.

The final offer can only be provided on evaluation of your Research Proposal with respect Host Institution etc. by the Expert Committee, securing the minimum score in evaluation and also the availability of seats under INSPIRE Fellowship in that year.

Without confirmation of your PhD admission and non-submission of your research proposal within the validity period of one year (365 days), the validity of your Provisional Offer will be expired automatically. On expiry of this offer, the online portal for submission of PhD details shall be closed in that case and then you will not be able to submit the documents. There will be no scope for giving any extension on the validity of the Provisional Offer in any situation. Documents sent as Email attachment or any other mode will be treated as invalid and no actions will be taken from DST.

In the event of you having being found ineligible at any state in future for the award/eligibility for INSPIRE Fellowship due to any reason (including unintentional computer error or printer's devil etc) this will be deemed withdrawn.

Dr. S. Mallikarjuna Babu
Scientist 'C'

Durga Apparao Bethala

C/O : BETHALA APPARAO RAMAYYA

Address : 1/11, MBPT NEW COLONY NADKARNI PARK, WADALA EAST.

City : Mumbai City

State/UT: MAHARASHTRA - 400037

This is a Computer Generated Offer Letter, No Signature is required



GENERAL INSTRUCTIONS

Date: 31 March, 2017

Dear Durga Apparao Bethala,

Please note that these documents are necessary to upload at INSPIRE Online Webportal only for final Selection under INSPIRE Fellowship

Following documents need to be uploaded in online portal <http://online-inspire.gov.in/> for further processing.

1. PhD admission Letter. (By which you got the confirmation about your PhD admission from your Host Institute Administration (Registrar/Comptroller/Dean/Director)). Please note that Admission Letters/Joining Letters endorsed/issued by Supervisor/Heads of Departments will not be accepted.
2. Endorsement Certificate (Template available in your dashboard in online portal).
3. Fee receipt (if deposited).
4. C.V. of the Supervisor.
5. Approved Research Topic and its detailed write-up which will be pursued during the PhD programme under this Fellowship.

No change in Research Proposal would be entertained in later date once submitted or Fellowship offered.

Please don't send any of these documents by post, any hard copy will not be entertained at this end.

This is for your kind information and necessary action.

Thanking you.

Dr. S. Mallikarjuna Babu
Scientist 'C'

This is computer generated document and does not require any signature. In case of any discrepancy please contact Program Division.



Newton-Bhabha PhD placements programme 2020-21

No. DST/INSPIRE/NBHF/2020/5

Dated.23.03.2021

Subject: Award Letter of Short-Term Research Internship for INSPIRE Fellows under Newton-Bhabha programme India (DST) and UK (British Council)

Dear Applicant,

Based on the announcement call and your application to the aforesaid subject, we are glad to inform you that the Joint panel for selection has recommended your name for Short-Term Research Internship (for 2-4 months) at various Universities/ Institutes at UK. However, you have been recommended for:

Name	Duration (Months)	UK Host Institute	UK Host Supervisor
Ms. Durga Bethala (IF170349)	4	Durham University	Dr Olena Riabinina

For this visit to UK for carrying out aforesaid Short-Term Research Internship the following payments shall be done from the Newton-Bhabha Program at respective Agency/Country:

a) DST/ India

- Shortest air-fare by Air-India in the International route. However, the domestic portion of the Journey needs to cover by Road/ Train (As per Government of India norms).
- Visa fee and overseas Travel Insurance Actual Cost. (As per Government of India norms).
- 90% shall be paid in advance through your present Institute and remaining on settlement after completing the training and receiving the Utilization certificate from Home Institute.

b) British Council/ UK

- Monthly stipend to cover meals, accommodation and domestic travel, etc and Bench Fee (as per Actual).

The Visit shall be expected in between April 2021 and December 2021.

You may please communicate the following information to DST within 10 days to process your financial part of this visit, scanned copies by email to sm.babu@gov.in and hard copy to Dr. S. Mallikarjuna Babu, INSPIRE Division, Department of Science and Technology, Ministry of Science and Technology, Technology Bhavan, New Delhi-110016.

1. Approval/ Endorsement from your Head of Institution/ Registrar of the University.
2. Acceptance of the Offer from your side.



Newton-Bhabha PhD placements programme 2020-21

Please also keep the following points noted for such visit:

- You must inform your Host Institute that you have been awarded the Newton Bhabha PhD placement grant and also that you wish to confirm your visit. Department of Science & Technology will not inform your Host institute of your application's success.
- Please address any questions you may have regarding the practical arrangements for your visit to your host institution.
- In any case the change in the total duration of stay will not be allowed. If you stay more than the permitted period, any more grant for that excess period will not be considered to release by DST.
- You must also inform us immediately about any changes in your contact email address. We send important information regarding your grant mainly by email. Please make sure that you regularly check your junk email also, because your email account filters may assign emails coming from the address not on your contact list there.

Dr. S. Mallikarjuna Babu,
Scientist-C,
Department of Science and Technology.

To,
No. DST/INSPIRE/NBHF/2020/5
Ms.Durga Bethala
C/o Dr.Shashikant P Vaidya
Department of Microbiology,
Haffkine Institute for Training, Research and
Testing, Acharya Donde Marg, Parel, Mumbai- 400012.

डॉ. मल्लिकार्जुना बाबू / Dr. S. Mallikarjuna Babu
वैज्ञानिक 'सी' / Scientist-C
विज्ञान और प्रौद्योगिकी विभाग
DEPT. OF SCIENCE AND TECHNOLOGY
भारत सरकार
नई दिल्ली-110002

Copy to
No. DST/INSPIRE/NBHF/2020/5
Dr Shashikant P Vaidya
Department of Microbiology,
Haffkine Institute for Training, Research and
Testing, Acharya Donde Marg, Parel, Mumbai- 400012.

No. DST/INSPIRE/NBHF/2020/5
Copy to:
The Director,
Haffkine Institute for Training, Research and
Testing, Acharya Donde Marg, Parel, Mumbai- 400012.

13 September 2021

DURGA APPARAO BETHALA
C/O Dr. Shashikant Vaidya, Clinical Pathology Department
Haffkine Institute For Training Research And Testing
Acharya Donde Marg, Parel
Mumbai
400012
India

Student ID Number: 000979989
Durham ID 528184

Dear Miss. Bethala,

DEFERRED OFFER

I am delighted to inform you that your application to study at Durham University as a postgraduate student has been successful. This letter contains very important information concerning your offer; please read it carefully. You must also read the additional information relating to your offer and preparing to study at Durham University which can be found on our website at www.durham.ac.uk/postgraduate/offerinformation

The summary details of your offer are as follows:

Programme	NP Visiting Research - Biological & Biomedical Sciences YAC130
Field Of Study	Drosophila fly model
Supervisors	Dr Olena Riabinina and Dr Karrera Djoko
JACS Code	C100 Biology
ATAS Statement	The main research aim of this placement is to develop a Drosophila model to study blood-brain barrier (BBB) integrity. Matrix metalloproteinases (MMP1 and MMP2) are crucial factors that can digest basement membrane proteins and Tight Junction proteins (TJ), thus affecting the integrity of the BBB in Drosophila, leading to virus invasion. Reactive Oxygen Species (ROS) directly downregulate TJs and indirectly activate MMPs that promote the opening of the BBB in murine microglial cells.
Department / School	Biosciences
Plan Of Study	Full Time
Start Date	13 September 2021
Duration	4 month(s)
End Date	12 January 2022 (end of supervised study)
Conditions	* Your offer is unconditional.
Fee Status	Overseas
Fees	£400 for academic year 2021/22.

ATAS CERTIFICATE



Foreign, Commonwealth
& Development Office

Atas reference: 294486
Certificate identifier: gyfxiw9ppc1a
Issued On: 27/09/2021
Expires On: 27/03/2022
Full name: Miss DURGA BETHALA
Date of Birth: 26/05/1992
Nationality: Indian
Passport number: N4161099
Passport expiry date: 19/10/2025
University/Research institution name: Durham University

You applied for ATAS clearance on 06/08/2021 to study Other Biology (CAH03-01-02) at Durham University.

You stated that your thesis, area of research, or the modules of your taught Postgraduate course would be:

"Dengue virus transmitted by Aedes species mosquitoes, thrives mainly in tropical zone of the world. Global warming has caused rise in temperature in colder temperate zones, exposing the dengue virus to population in these regions. The dengue virus invasion in brain occurs at the final stage of infection i.e. Dengue Shock Syndrome, is associated with high mortality. This project is important because it tackles the urgent and poorly understood question of how the Dengue virus affects brain function which eventually causes death in severely infected patient. In the absence of clinically approved drug or vaccines, viral diseases like dengue primarily depends on the supportive and symptomatic management. A big part of this management strategy focuses on the co-pathological condition that arise beyond the preliminary pathology. Neurological manifestations of dengue virus may occur during or after acute infection, and includes a breach of blood-brain barrier (BBB). Understanding the mechanism of BBB breach shall allow us to design better disease management strategies. I have so far studied initial dengue pathogenesis using in-vitro mice cell line models. But the Drosophila fly brain model could be much better model to study viral neuropathogenesis. The fly model readily mimic the live physiology of mice or human because of its genetic predisposition. These disease models are much simple and easier to handle, citing the low-cost expenditure to perform intricate assay on fly models. The aim of this placement is to develop a fly model to study BBB integrity. This development of neurobiology model in fly would be achieved by crossing different fly variant to generate oxidative stress, which would eventually disrupt drosophila BBB. The results from this experiment would be compared with the actual infection of dengue virus infection on fly. The mouse and the fly models, together, will

ATAS CERTIFICATE



Foreign, Commonwealth
& Development Office

thus allow the development of efficient drugs in management of dengue encephalitis in humans."

I am pleased to inform you that your ATAS application was successful.

You will need to include this ATAS certificate with your other supporting documents when you make your visa application or if you are a visitor, before you begin your research.

This certificate is only valid for the named individual, University / Higher Education Institution / Research Institution and subject areas stated above. It is valid for six months from the date of issue. However, if you wish to study or undertake research at another University / Higher Education Institution / Research Institution, change your course / area of research or extend your course or research by more than three months, you will need to apply for another ATAS certificate. You can do this via the ATAS website <https://www.academic-technology-approval.service.gov.uk>.

You should be aware that submitting forged or fraudulently obtained documents, including a fake ATAS certificate, in support of a visa application is a serious offence. You may face a fine, prison and a ban of up to ten years on making a further visa application.

The ATAS Team.
Foreign, Commonwealth & Development Office.

Research Papers and Poster Presented

Poster

1. Presented poster on the topic titled 'Essential oils: A medicated gargle against H1N1', Durga Bethala, Sweta Kothari, Pavankumar Todkar, Rashmi Yadav, Surabhi Rode in Virocon-2017 organised by Indian Virological Society in Nitte University, Mangalore.
2. Poster entitled, 'Mitochondria-targeted liposomes of metformin for improved anticancer activity: preparation and evaluation', Juber Pendhari, Hemali Savla, Durga Bethala, Shashikant Vaidya, Ujwala Shinde, Mala Menon, Secured 2nd prize at CRSIC's19th International e- Symposium 2021.
3. Presented oral paper presentation on topic titled 'Overexpression of iNOS and dmMMP disrupts the glial barrier in adult *Drosophila*', Durga Bethala, Shashikant Vaidya; in the National Conference on the International Symposium on Translational Research, held on 8th and 9th September, 2022 at Vijnana Bhavan, University of Mysore, Mansangangothri, Mysuru.

Papers

1. Identification of ligand-receptor interaction across the blood-brain barrier in dengue pathogenesis, Shashikant Vaidya, Durga Bethala, BioMedicalCentral Infectious Diseases, May 2020, 20 (Supp 11):324, Page 6, <https://doi.org/10.1186/s12879-020-05038-y>, BMC Infectious Diseases 2020 (Suppl 1):324, ISSN:1471-2334.
2. Mitochondria Targeted Liposomes of Metformin for Improved Anticancer Activity: Preparation and Evaluation, Juber Pendhari, Durga Bethala, Shashikant Vaidya, Ujwala Shinde, Mala Menon, 2022, SSRN, JDDST-D-22-01240.
3. Investigating the Molecular Response of Microglia to Lipopolysaccharide-induced Neurotropic Activation: Implications for Blood-Brain Barrier Breakdown, Durga Bethala, Shashikant Vaidya; International Journal of Applied Chemical and Biological Sciences (2023), 4(1), 18-27 ISSN- 2582-788X.
4. Neurotropic Virus Infections and their Impact on Brain Endothelial Cells: Implications for Dengue Virus Pathogenesis (Communication)
5. A comparative analysis on tropical neurotropic virus causing blood-brain barrier disruption (Communication)



Haffkine Institute for Training, Research and Testing

Acharya Donde Marg, Parel, Mumbai - 400 012. Tel: 022 - 24160947, 24160961, 24160962. Fax : 022 - 24161787 email : haffkineinstitute@gmail.com, website: www.haffkineinstitute.org



ESSENTIAL OIL: A MEDICATED GARGLE AGAINST H1N1

Sweta T. Kothari, Pavankumar Todkar, **Durga Bethala**, Surabhi Rode.
Department of Virology, Haffkine Institute, Mumbai. durga.bethala@gmail.com

ABSTRACT

pdm 09 H1N1 Influenza A is a continuously evolving form of viral infection, prevalent in South Eastern Asia, and a rise in morbidity and mortality has been observed inspite of presence of a vaccine regimen. There arises a need to develop a safe, affordable and potent anti-viral agent against pdm 09 Influenza A H1N1. Some essential oils have demonstrated effective anti-microbial and anti-viral properties in experimental conditions. However, most of these studies tested the liquid-oil phases, which are generally less practical and are potentially toxic for oral applications. With this rationale thyme, tea tree, lemon and lavender essential oils were made into stable formulations in aqueous medium. In order to target the upper respiratory tract, a potent o/w (oil in water) emulsion based gargle formulation complied under British Pharmaceutical Codex (BPC) was developed by homogenization. *In vitro* cytotoxicity analysis of essential oils were carried out on MDCK cell line using MTT assay and virucidal activity was determined by amido black staining. CC₅₀ values of the formulation of lemon oil, lavender oil, thyme and tea tree oil were found to be 0.063% v/v, 0.03% v/v, 0.015% v/v and 0.007% v/v respectively. The reduction of viral infectivity by these essential oils was measured by identifying the reduction in cytopathic effects using Spearman Karber method. A comparative study of antiviral activity of these emulsion based gargle formulations against pdm 09 Influenza A H1N1 was evaluated on real-time PCR. From the present study, it could be concluded that lemon oil and lavender oil based gargles had a higher log reduction values 2.37 and 2.34 respectively, than thyme oil and tea tree based gargles showing 1.67 and 1.63 log reduction respectively. Our study lays the foundation for larger and more intensive pharmaceutical studies in order to develop a target specific gargle formulation.

INTRODUCTION

- Belonging to Orthomyxoviridae, Influenza A virus can infect humans and cause annual influenza epidemics resulting in significant global morbidity and mortality.
- pdm 09 H1N1 strain represents a quadruple reassortment of two strains of swine strains (30.6% North American swine and 17.5), 34.4 % North American avian strain and 17.5% Human influenza virus strain.
- Vaccines are the best option for the prophylaxis and control of a pandemic; however, the lag time between virus identification and vaccine distribution indicates that in the short-term, antiviral therapy is vital to control the spread of influenza.
- Despite intense efforts by pharmaceutical companies, relatively few antiviral drugs are available, in contrast to the plethora of antibiotics in clinical use. Of the drugs available, widespread use of certain anti-virals have led to emergence of drug resistant virus strains, while many of the drugs have been found to be toxic in humans.
- Essential oils enjoy remarkable popularity as an anti-inflammatory, anti-microbial, anti-viral and as an natural preservatives in many pharmaceutical and cosmetic products.
- Oils of Lavender (*Lavandula officinalis*), tea tree (*Melaleuca alternifolia*), lemon (*Citrus limonius*), thyme (*Thymus vulgaris*) that have been used in alternative medicine for respiratory ailments may present with antiviral activity when designed in suitable dosage form.

OBJECTIVES

- Bench-to-bedside formulation of essential oil in liquid emulsion dosage form (gargle) for oromucosal usage against pdm 2009 H1N1 influenza virus in cell culture.
- Establishing a stable essential oil gargle by characterization of its physical stability parameters.
- Determining its half maximum cytotoxic concentration of essential oil gargle and evaluating its virucidal activity against H1N1.

METHODOLOGY

- **Formulation of Essential oil gargle:** Essential gargle was prepared by Forbes Bottle method followed by homogenization by mechanical stirring. A 2% v/v essential oil (internal phase) was prepared by sequential dispersion of Polysorbate 80 with further addition of double strength chloroform water, mannitol and external phase purified water (q.s).
- **Cells and Virus propagation:** Madin-Darby canine kidney cells were maintained in Eagle's Minimum Essential Medium supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) at 37 °C with 5% CO₂.
- **Cell Cytotoxicity MTT Assay:** MDCK cells (2 x10⁴ cells/well) were seeded in 96-well plates and incubated at 37 °C and 5% CO₂ overnight. MTT was added to each well, incubated for 1 hour and absorbance was recorded at 570nm. The concentration of essential oil gargle that caused the death of 50% of the cells was defined as the CC₅₀ of that oil.
- **Antiviral assay of essential oil gargle:** pdm 2009 H1N1 virus was neutralized with essential oil gargle for 2 mins. A serial dilution (10-1 to 10-8) of the treated virus was overlaid on MDCK cells seeded in 96-well plate. This plates were incubated for 4-6 days and then stained with amido black. TCID₅₀ was calculated using Spearman-Kärber method.
- **Quantitative analysis of essential oil gargle:** TaqMan qRT-PCR was used to quantify the presence of viral M1 gene in the media of the MDCK cells after influenza virus infection (virus control) and post treatment with essential oil gargle virus.

RESULTS

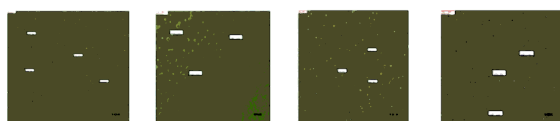


Fig.1 : o/w essential oil gargle of different oils with kinetically stable globules size ranging from (0.68µm to 4.6µm) macro-emulsions.

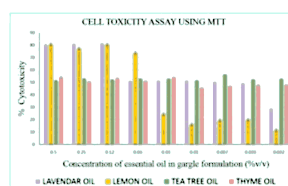


Fig.2: Determination of % cytotoxicity of the essential oil in gargle

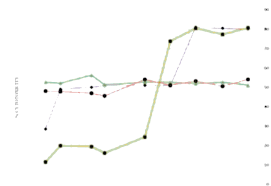


Fig.3: CC₅₀ of lemon (0.063%), lavender (0.03%), tea tree (0.015%) and thyme (0.007%) oils (all v/v)

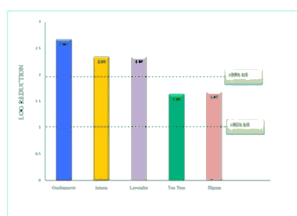


Fig.4: Inhibitory effect of essential oils in comparison with oseltamivir carboxylate on influenza viral infection in MDCK cells.

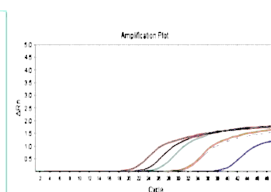


Fig.5: Graphical representation of virucidal activity of essential oil gargle on influenza A H1N1 virus by real-time RT-PCR

■ Lemon oil ■ Lavender oil ■ Tea Tree oil ■ Thyme oil ■ Oseltamivir carboxylate ■ Virus control

CONCLUSION

- Essential oil inactivated pdm 2009 H1N1 influenza virus resulting in more than two log reduction of the titer by in MDCK cells.
- The essential oil gargle formulation of lavender oil and lemon oil showed promising results of more than 99% virucidal activity as compared to tea tree and thyme oil depicting 90% virucidal effect.
- Apart from its pleasant soothing effect this gargle is alcohol free as against marketed preparation.
- The physical stability study revealed a stable emulsion based gargle with no signs of flocculation, coalescence, phase inversion and breaking for more than a month at room temperature.
- This preliminary study of gargle formulation needs further investigation about characterization of active compounds and their specific mechanism against influenza virus.
- Given the urgent need for new interventions to tackle the spread of influenza infections, essential oil based gargle appears to be a promising option as a supplemental strategy to currently available anti-influenza therapies.

ACKNOWLEDGEMENT

We thank Dr. Nishigandha Naik, Director Haffkine Institute for Training, Research and Testing for her constant inspiration. Authors thank M's R.K. Aroma, Mumbai, for the kind gift of the essential oils.

REFERENCES

- Elie K. Barbour et al., Safety and antiviral activity of essential oil against avian influenza and new cattle disease viruses, Department of animal and veterinary science.
- Jürgen Reichling et al., Essential oils of aromatic plants with antibacterial, antifungal, antiviral and cytotoxic properties- an overview, Institute of Pharmacy and Molecular Biotechnology, Department of Biology, 2009.
- Carson et al., *Melaleuca alternifolia* (Tea Tree) oil: A review of antimicrobial and other medicinal properties. Clinical Microbiology Reviews 2006; 19:50-62.
- Selvarani Vimalanathan, James Hudson, Anti-influenza virus activity of essential oil and vapors

Overexpression of iNOS and dmMMP disrupts the glial barrier in adult *Drosophila*

- **Durga Bethala¹**, DST-INSPIRE (SRF), Department of Clinical Pathology, Haffkine Institute for Training, Research and Testing, Mumbai. **(Presenting Author)**
- Shashikant Vaidya¹, Assistant Director & Head, Department of Clinical Pathology, Haffkine Institute for Training, Research and Testing, Mumbai.

Abstract:

It is well known that the central nervous system is shielded from circulating immune cells by the blood-brain barrier (BBB). During pathogenic infections and autoimmune diseases, neurological manifestations like glial inflammation occurs which effects the stability of the BBB structure and brain homeostasis. Here, we developed a *Drosophila* model to study the effect of reactive oxygen species and matrix metalloproteinases on blood brain barrier integrity and its link for barrier inflammation. The fly model readily mimics the live physiology of mice and human because of its genetic predisposition also it has lower maintenance cost. By crossing the different *Drosophila* glia genetic lines, we developed different fly transgenic lines having super-expressed (UAS-mac-NOS), moderately expressed (UAS-NOS-NOS) and low-expressed (UAS-NOS-RNAi) iNOS in fly brain. We observed that moody-mac-NOS lines have upregulation in iNOS and dmMMP when compared to repo-mac-NOS and NP6293-mac-NOS lines. The moody-mac-NOS *Drosophila* brain immunostaining, transmission electron microscopy results show a loss of Nr4 from the outermost transmembrane boundary suggesting the loss of blood-barrier integrity. We observed that overexpression of iNOS and dmMMP (*Drosophila melanogaster* MMP) does has significant impact on the loss of Nr4 protein from the transmembrane region of the adult *Drosophila* brain. This suggest that alleviated levels of iNOS have impact on the moody-mac-NOS *Drosophila* BBB integrity and dmMMP has any role in aiding the barrier impairment. These findings have implications not only for invertebrate epithelia and barrier functions, but also for understanding the effect of neurotropic virus interactions in the mammalian nervous system.

Presented at National Conference on the International Symposium on Translational Research, held on 8th and 9th September 2022 at Vijnana Bhavan, University of Mysore, Mansagangothri, Mysuru.

Article Identifier: <https://identifier.visnav.in/1.0001/ijacs-23b-29001/>

Investigating the Molecular Response of Microglia to Lipopolysaccharide-induced Neurotropic Activation: Implications for Blood-Brain Barrier Breakdown

Shashikant Prabhakar Vaidya and Durga Apparao Bethala *

Clinical Pathology Department, Haffkine Institute for Training, Research and Testing, Acharya Donde Marg, Parel, Mumbai – 12, Maharashtra, INDIA.

* For correspondence: durga.bethala@gmail.com

Received on: 28 December 2022

Published on: 30 January 2023

ABSTRACT

We investigated the molecular response of microglia, a key player in brain defense, to neurotropic activation by lipopolysaccharide. We found increased expression of pro-inflammatory and regulatory signaling markers, as well as blood brain barrier (BBB) breakdown. Specifically, we observed significant increases in TNF- α (>4-fold) and IL-1 β (>8-fold), which indicate inflammation, and in IL-6 and MCP-1, which signal leukocyte recruitment and infiltration. We also saw an increase in MMP-2 expression and a decrease in Occludin expression, indicating BBB breakdown. However, we found no changes in MMP-9 or its inhibitor TIMP-1. Our study is the first to uncover the molecular mechanisms of early immune response and its effect on barrier proteins of the microglial cell, and will be useful for understanding the brain's immune response to infectious diseases.

Keywords: Microglia, tight junction protein, cytokine, blood-brain barrier

1. INTRODUCTION

Microglia, the resident immune cells of the central nervous system (CNS), play a crucial role in maintaining the homeostasis of the brain and responding to various pathological conditions. Microglia, the resident immune cells of the CNS, play a crucial role in defending against infection and inflammation in the immune-privileged environment of the CNS. As members of the macrophageal lineage, microglia are uniquely equipped

to respond to and combat pathogens and other immune challenges within the CNS [1]. Microglial cells possess the capability to migrate in response to even the smallest insult or injury in the CNS, specifically the brain [2]. To gain an understanding of the microglial cells' immune response, many models, such as three-dimensional models based on collagen, have been studied using both animal and cell-based methods [2,3]. After encountering foreign materials such as

Lipopolysaccharide (LPS), microglial cells become activated and release a range of cytokines and chemokines necessary for restoring immune balance. These stimuli can be either short-lived or long-lasting injuries, resulting in increased neuroinflammation. Microglial cells also play a significant role in the neuronal inflammasome, controlling processes such as apoptosis and cell death [4].

The blood-brain barrier, or BBB, is a complex structure that acts as a barrier between the bloodstream and the brain. It is made up of specialized cells called endothelial cells, which line the blood vessels in the brain, and the tight junctions between these cells. These structures work together to create a barrier that is both physical and immunological in nature. The BBB is highly selective, allowing certain molecules to cross the barrier while preventing others from passing through. This is accomplished through a combination of passive diffusion and active transport. Passive diffusion is the natural movement of molecules from an area of higher concentration to an area of lower concentration. However, the BBB prevents this process for many substances, including gases and ions, that might be harmful to the brain. On the other hand, the BBB allows certain essential nutrients to cross the barrier through active transport. This is a process by which molecules are moved across the barrier with the help of specific transporters, which recognize and bind to specific molecules. This enables the brain to receive the essential nutrients it needs to function properly while keeping harmful substances out. Overall, The blood brain barrier plays a crucial role in maintaining the delicate environment of the central nervous system, by regulating the transfer of molecules between the blood and the brain [5]. In addition to microglial cells, the blood-brain barrier is composed of various other elements such as astrocytes, podocytes, pericytes, pinocytic vesicles, and tight junction proteins [6]. The

glial cells are the ones that react to any small changes in the microenvironment of the central nervous system. It has been observed that changes in the levels of cytokines can affect the permeability of the blood-brain barrier by regulating the tight junctions in the endothelial cells present in the brain's blood vessels [7].

BV-2 cells are a type of mouse microglia that have been genetically modified to be immortalized, which means they can continue to grow and divide in laboratory conditions [8,9]. These cells are widely used in scientific research for studying various pharmacological and immunological processes [10]. They are known to produce a variety of proteins and chemicals, such as chemokines, that are involved in maintaining the immune system and the blood-brain barrier (BBB). Some of the key cytokines produced by BV-2 cells include TNF- α and IL-1 β , which have pro-inflammatory roles, while others like IL-6 are involved in the recruitment of immune cells to sites of injury [11]. BV-2 cells are also known to secrete enzymes called metalloproteinases (MMPs)[12] and a protein called Occludin [13], which are thought to play important roles in the breakdown of the BBB.

In the present study, we aimed to investigate the early immune response of microglia under laboratory defined conditions, using the BV2 cell line as a model. We standardized the stimulation of these cells and measured their response in order to establish a well-defined model of microglial activation. Our results demonstrate that this model can be useful for studying various CNS inflammation and infectious conditions, as it allows for the controlled elicitation of immune response in microglia. We propose that this model can provide valuable insights into the mechanisms of microglial activation and aid in the development of new therapeutic strategies for CNS disorders.

2. METHOD AND MATERIALS

2.1. Cell characterization and maintenance for immune response studies

The Murine microglial cell line (BV2) was chosen as the substrate for the study because it is a well-established model for studying the immune response of microglia. These cells were grown in a specific type of medium, Dulbecco's Modified Eagle Medium (DMEM, Himedia), which contains certain chemicals and supplements that help to support the growth of the cells. Additionally, the medium was supplemented with 7.5% sodium bicarbonate (Gibco) and 10% Fetal Bovine Serum (FBS, Gibco) which helps to provide the necessary nutrients and growth factors for the cells. The cells were grown in an incubator at 37°C with 5% CO₂ to mimic the conditions of the body. Once the cells reached confluency, they were sub-cultured or propagated by using mechanical disruption procedures or scraping and subsequently centrifuged at 1500rpm for 10 minutes. The cells were then split in the ratio of 1:2 to 1:4 as per the requirement for the experiment.

2.2. Effects of lipopolysaccharide stimulation on the BBB using BV2 cells

BV-2 cells are a type of cell culture that are commonly used in research to study the blood-brain barrier. In this study, the researchers seeded BV-2 cells in a 25 cm² flask and allowed them to grow overnight in a culture medium called DMEM, which is enriched with 2% fetal bovine serum (FBS). The next day, the cells were treated with a stimulus called Lipopolysaccharide (LPS), which is a type of molecule that can induce inflammation. The LPS was added to the culture medium at a concentration of 100 ng/ml and the cells were incubated with this stimulus for 4 hours. To ensure a fair comparison, an identical set of control flasks were also set up and treated with culture medium alone, without the addition

of LPS. This will allow the researchers to observe the effects of LPS specifically, without the influence of other variables [3].

2.3. RNA isolation and cDNA synthesis for gene expression analysis in BV2 cells

In order to analyze gene expression, the researchers first extracted total RNA from the cells using the RNeasy Mini kit (Qiagen, India). The kit is designed for the isolation of high-quality RNA from a wide range of samples, including cells. The process began by lysing 106-108 cells and then extracting the RNA as per the manufacturer's protocol. After the extraction, the RNA was converted to cDNA (Takara Bio, India) using a reverse transcription process. The cDNA was then stored at -80°C until it was ready to be used for gene expression analysis. This step is important because it enables researchers to analyze the genes that are being transcribed in the cells, which can provide valuable insights into cellular processes and responses.

2.4. qPCR analysis of BBB breakdown and immune response in BV2 cells treated with lipopolysaccharides

In the present study, gene expression was divided into three distinct categories in order to better understand the specific changes occurring in the cells being studied. The first category, labeled as "Inflammatory," includes a group of genes that are known to play a role in initiating and exacerbating inflammation within the body. These genes include Interleukin-1 beta (IL-1 β), Tumor Necrosis Factor alpha (TNF- α), Interleukin-10 (IL-10), Inducible nitric oxide synthase (iNOS), Interferon gamma (IFN- γ), and Interleukin-18 (IL-18). The second category, labeled as "Regulatory," includes genes that play a role in regulating the immune response, such as Monocyte chemoattractant protein-1 (MCP-1) and Interleukin-6 (IL-6). The final category, labeled as "BBB

breakdown," includes genes that have been linked to the breakdown of the blood-brain barrier, such as Occludin, Matrix metalloproteinases (MMP2, MMP9) and Tissue Inhibitor of Metalloproteinase (TIMP1). To analyze the expression of these genes, the researchers used a technique called real-time PCR, which allows for the quantification of specific RNA molecules in a sample. They used a kit from Takara Bio (India) and followed the primers and cycling conditions that were described in previous studies [14,15]. A housekeeping gene, GAPDH, was also used as a control to ensure that the changes in gene expression were specific to the genes of interest. To obtain accurate results, three separate BV2 flasks were used for each gene and the ΔCt value, which represents the difference in cycle threshold between the target gene and the housekeeping gene, was calculated. Fold change was then determined using the $\Delta\Delta Ct$ method and a change was considered significant if the $\Delta\Delta Ct$ value was greater than 2 [16].

3. RESULT AND DISCUSSION

3.1. Morphological changes in BV2 cells following lipopolysaccharide stimulation

Non-stimulated or unstimulated BV2 cells, a commonly used microglial cell line, appeared diverse in terms of shape and structure. Without any external stimulus, the cells displayed a range of morphologies, from spherical to ameboid, and some cells even had protrusions. However, upon exposure to LPS (100ng/mL), a well-established pro-inflammatory stimulus, the cells underwent a significant change in their appearance. The protrusions on the cells were greatly reduced and the overall shape of the cells became more spherical or flattened. Additionally, if the cells had "spikes" present, they were observed to be thickened and shortened as shown in Figure 1. This change in cell morphology after LPS exposure is likely a result of the inflammatory response induced in the cells.

3.2. Evaluation of GAPDH as a housekeeping gene in Lipopolysaccharide-exposed BV2 cell

In order to assess the impact of LPS (lipopolysaccharide) exposure on cellular gene expression, we performed a series of experiments on BV2 cells, a commonly used model for studying microglial activation. One of the initial steps in these experiments was to determine an appropriate reference

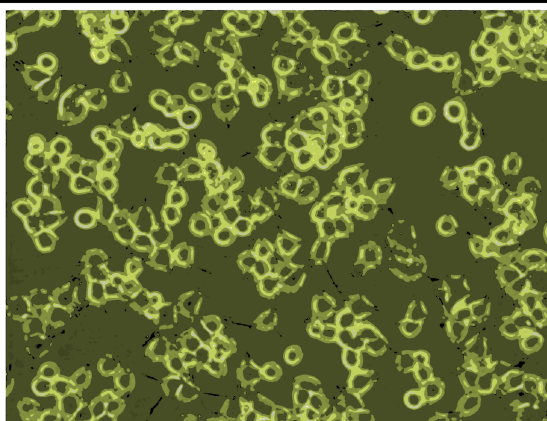


Figure 1. BV2 (Murine microglial) cells grown in DMEM with FBS, cells show heterogeneous morphology with presence of prominent 'spikes' or protrusions.

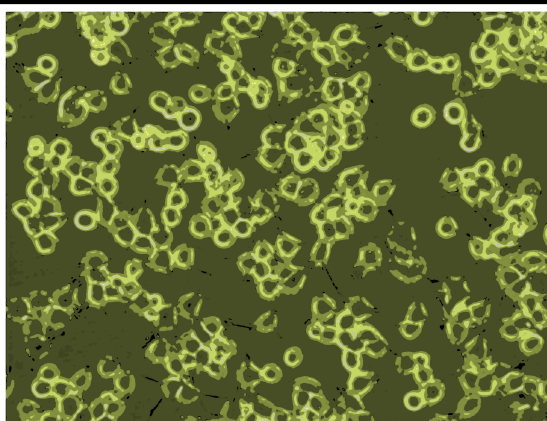


Figure 2. BV2 cells 4 hours after exposure to LPS (100ng/mL), cells show distinct flattening and increased appearance of spherical cells, with reduction in the number of spikes

gene, or "housekeeping" gene, to normalize our data. To do this, we compared the expression of the gene encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in untreated and LPS-exposed cells after a 4 hour exposure period. By analyzing the Ct value, a measure of the amount of starting template in the PCR reaction, we observed a fold change of 1.03 between the two groups. This relatively small difference in GAPDH expression between the two groups indicates that it would be a suitable reference gene for normalizing our data. As a result, all further gene expression measurements were normalized to the respective GAPDH levels in order to accurately compare changes in expression between the untreated and LPS-exposed cells.

3.3. Analysis of pro-inflammatory, regulatory, and BBB breakdown markers

In the present study, we exposed BV2 cells to LPS (100ng/mL) for a period of 4 hours in order to induce an inflammatory response. Our observations showed a shift in favor of pro-inflammatory markers, with the primary marker being TNF- α as shown in Table 1. However, it's worthy to note that this early response did

not elicit any signs of oxidative stress related apoptosis, as seen by an insignificant rise in the mRNA levels of iNOS. This led us to investigate downstream markers in order to better understand the specific changes that were occurring in the cells. Upon further examination, we observed a similar shift in the regulatory markers under investigation towards inflammation and chemotaxis dependent recruitment of neutrophils and other mononucleocytes as shown in Table 2. In particular, the increment in the expression of IL-6, which is a marker indicated for increased vascular permeability, from microglial cells led us to assess the levels of gelatinases and occludin associated with the breakdown of the blood-brain barrier (BBB). In agreement with our hypothesis, we indeed observed the markers to be regulated in favor of this compromise. Though we had expected both gelatinases under evaluation (MMP-2 and MMP-9) to increase, we did not observe any significant rise in the MMP-9 mRNA levels as shown in Table 3. We presume that our analysis period of 4 hours was insufficient to detect this increase, since its inhibitor TIMP-1 had already started being down-regulated due to LPS induction. This suggests that a longer period of LPS exposure may be necessary in

Table 1. Gene expression of cytokines involved in inducing inflammation after LPS exposure for 4 hours.

Target gene	ΔCt Base line (Mean $\pm$ SD)	ΔCt LPS (Mean $\pm$ SD)	Fold Change
IL-1 β	10.69 $\pm$ 0.89	9.34 $\pm$ 1.66	2.57
TNF- α	5.93 $\pm$ 1.29	3.88 $\pm$ 1.64	4.16
IL-10	10.23 $\pm$ 0.22	11.92 $\pm$ 0.43	-3.24
IFN- γ	8.33 $\pm$ 0.80	11.91 $\pm$ 0.87	-12.00
iNOS	7.84 $\pm$ 0.71	7.74 $\pm$ 0.96	1.07
IL-18	12.60 $\pm$ 0.59	12.26 $\pm$ 0.41	1.25

Table 2. Gene expression of chemokines involved in regulating inflammation after LPS induction for 4 hours.

Target gene	ΔCt Base line (Mean $\pm$ SD)	ΔCt LPS (Mean $\pm$ SD)	Fold Change
IL-6	11.35 $\pm$ 1.43	10.19 $\pm$ 0.97	2.23
MCP-1	5.40 $\pm$ 0.55	4.70 $\pm$ 0.45	1.62

Table 3. Gene expression of proteins involved in BBB compromise after LPS induction for 4 hours

Target gene	ΔCt Base line (Mean $\pm$ SD)	ΔCt LPS (Mean $\pm$ SD)	Fold Change
Occludin	8.28 $\pm$ 0.64	11.53 $\pm$ 0.45	-9.55
MMP-2	15.42 $\pm$ 0.54	12.39 $\pm$ 0.80	8.13
MMP-9	12.58 $\pm$ 0.76	13.42 $\pm$ 1.32	-1.79
TIMP-1	11.81 $\pm$ 0.21	15.45 $\pm$ 0.71	-12.49

order to fully observe the effects of MMP-9 on BBB breakdown.

Lipopolysaccharide (LPS) is a substance known to have pro-inflammatory effects on the body. Researchers have used in-vitro and ex-vivo models to study the immune response generated by LPS [1]. Microglia, a type of immune cell in the brain, play a crucial role in this response by secreting various cytokines to other cells such as endothelial cells, astrocytes, and podocytes. This helps to contribute to the maintenance of the blood-brain barrier (BBB). Recently, a three-dimensional collagen construct of microglial cells from mice has been created to study the activation of these cells due to LPS. In the present study, the researchers aimed to understand and identify the immune mechanisms involved in three categories: inflammation, regulation,

and BBB breakdown. To do this, they used BV-2 cells, which are microglial cells of murine origin [3]. Cytokines have been found to play a role in various forms of neurodegeneration, and are induced in response to brain injury, having diverse actions during cellular injury and repair [17].

The present study aimed to investigate an early microglial response to stimuli in relation to inflammation, immune regulation, and blood-brain barrier (BBB) breakdown. To do this, the researchers activated microglial cells for 4 hours using Lipopolysaccharide (LPS) [17]. At the end of this induction period, they observed a pro-inflammatory response, indicated by an increase in the cytokine TNF- α and a decrease in the cytokine IL-10 [20]. These two cytokines have opposite effects on the body, with TNF- α

being neurotoxic and IL-10 being protective. The researchers also observed that the anti-microbial cytokine IFN- γ was down-regulated more than ten-fold [19]. They suggest that this may be due to the short induction period or the presence of another cytokine, IL-15, which has been known to down-regulate the expression of IFN- γ [21]. Additionally, there was no evidence of oxidative stress to the cells after LPS insult within the exposure period, as observed by an insignificant upregulation in the expression levels of iNOS. This differs from previous studies that have reported a significant upregulation in iNOS levels after longer exposure to LPS or higher concentrations of the polysaccharide [18]. The researchers also investigated the gene expression of IL-1 β , a potent signaling molecule that is normally expressed at low levels but is rapidly induced in response to local insults [10]. This makes it an optimal marker for the determination of inflammation in the cells in the present study. The researchers found that inflammation by LPS was confirmed by a significant increase in the mRNA levels of IL-1 β . Though IL-1 β alone might not contribute to apoptosis of the neuronal cells, it might act synergistically with other Th1 cytokines [10], and more importantly, it is implicated in activation of microglia and self-perpetuating neurotoxicity [23].

Our findings of increased TNF- α and IL-1 β gene expressions led us to investigate whether there was a corresponding increase in the expression levels of IL-18, which is known to enhance the expression of these two cytokines [24]. Although we did observe a relative increase in the mRNA levels of IL-18, it was not statistically significant. We hypothesize that the peak IL-18 gene expression may have occurred earlier than the end-time of the LPS exposure. Additionally, we also investigated the downstream effects of this inflammation chain by studying the induction of IL-10 gene expression by TNF- α . After 4 hours, we observed

that the negative feedback mechanism had not yet started, as indicated by a relative decrease in the mRNA levels of IL-10.

The blood-brain barrier (BBB) plays a crucial role in preventing the influx of most compounds from blood to brain. However, mechanisms that can cause the breakdown of the BBB include increased vessel permeability, leukocyte infiltration of the central nervous system (CNS), and loss of tight junction proteins. The proper function of BBB is dependent on the function of various cell types in the CNS, such as Pericytes, podocytes, astrocytes, and microglial [25].

In our study, we aimed to understand the regulatory mechanism in microglia that could lead to the breakdown of the BBB during brain inflammation. To do this, we investigated the gene expression of MCP1, a member of the C-C chemokine family that is secreted by microglial cells as an anti-viral defense mechanism. We observed a one and half fold increase in the levels of MCP1, which although not statistically significant, may be an early indicator of BBB breakdown. MCP-1 is known to regulate the infiltration of monocytes, T lymphocytes, and natural killer cells in the neuronal locales through chemo-attraction [26]. Additionally, we also found that vessel permeability is governed by IL-6, which is also known to play a role in amplification of leukocyte recruitment, contributing to local inflammatory responses. In our study model, we observed a two-fold increase in the IL-6 [27] gene expression levels after LPS induction, when compared to the baseline, indicating a shift towards the breakdown of the BBB in the microglial environment [28].

In our study, we placed equal importance on the function of tight junction proteins (TJ proteins) and matrix metalloproteinases, as they play crucial roles in

regulating the integrity of the BBB. TJ proteins are specialized multi-protein complexes located at the apical contact point between cells in epithelial and endothelial tissues, and act to seal the intercellular space, creating a permeability barrier required for transport processes [29]. Among these proteins, Occludin, a tetraspan membrane protein, plays a key role in regulating what enters and exits the neurovascular unit of the brain, and its expression is directly associated with the integrity of the BBB [30]. In the endothelium, proteolytic cleavage of occludin to inactive fragments, which is mediated by matrix metalloproteinases (MMPs), particularly gelatinases (MMP-2 and MMP-9), leads to barrier disruption. In our analysis, we observed a significant decrease in the genomic expression of Occludin, which would be a precursor to low protein levels and a compromised BBB. As a downstream investigation, we decided to study the expression of the gelatinases in a BV2 cell culture model.

Previous studies have shown that MMP-2 plays a role in the degradation of occludin and the alteration of BBB integrity during the early stages of ischemia, while MMP-9 and other factors contribute to further degradation and long-term changes [31]. Our study on LPS-induced inflammation evaluated the early responses at 4 hours of exposure. We found a significant increase in MMP-2 levels, while MMP-9 levels remained similar to the baseline. This is consistent with previous research that suggests MMP-2 plays a role earlier than MMP-9 [31]. As there was no significant change in MMP-9 mRNA levels, we also looked at the expression of its corresponding inhibitor TIMP-1 [32], which was significantly down-regulated, indicating that the low levels of MMP-9 may increase with longer exposure to an inflammation stimulant like LPS.

4. CONCLUSION

In conclusion, our study has shown that BV2 cells are a suitable model for investigating the effects of LPS-induced inflammation on the blood-brain barrier. By using these cells, researchers can study the microglia's role in the breakdown of the blood-brain barrier, as well as other conditions such as neurotoxicity, viral and bacterial brain infections, and immune responses in a controlled and consistent manner, as opposed to using animal models which can vary batch to batch.

5. ACKNOWLEDGEMENT

We express our gratitude to the Director, Haffkine Institute for Training Research and Testing, for valuable suggestions and encouragement. We acknowledge DST-INSPIRE Fellowship and Haffkine Institute for Training Research and Testing for providing financial assistance.

6. CONFLICT OF INTEREST

The authors have declared that there is no conflict of interest.

7. SOURCE/S OF FUNDING

The current research project was approved by Institutional Bio-Safety Committee (IBSC) vide Ref. HITRT/IBSC/2017/C-2.

8. REFERENCES

1. Haw R, Tong C, Yew A, Lee H, Phillips J, Vidyadaran S. A three-dimensional collagen construct to model lipopolysaccharide-induced activation of BV2 microglia, *J Neuroinflamm.* 11(2014) 134
2. Carbonell W. S., Murase S., Horwitz A. F. and Mandell J. W. Migration of perilesional microglia after focal brain injury and modulation by CC chemokine receptor 5: an in situ time-lapse confocal imaging study. *J. Neurosci.* 25 (2005) 7040-7047.

3. Henn A, Lund S, Hedtjarn M, Schrattenholz A, Porzgen P, Leist M. The suitability of BV2 cells as alternative model system for primary microglia cultures or for animal experiments examining brain inflammation. *Altx*.26 (2009) 83-94.
4. Walsh JG, Muruve DA and Power C. Inflammasomes in the CNS. *Nature Reviews: Neuroscience*.15 (2014) 84-97.
5. Joan Abbott N, Rönnbäck L and Hansson E. Astrocyte-endothelial interactions at the blood-brain barrier. *Nature Reviews: Neuroscience*. 7 (2006) 41-53
6. Miller D. Immunobiology of the blood-brain barrier. *J Neurovirol*. 5 (1999) 570-578.
7. Wong, D., Dorovini-Zis, K., Vincent, S.R. Cytokines, nitric oxide, and cGMP modulate the permeability of an in vitro model of the human blood-brain barrier. *Exp Neurol*. 190 (2004) 446-455.
8. Blasi, E., Barluzzi, R., Bocchini, V. et al. Immortalization of murine microglial cells by a v-raf/v-myc carrying retrovirus. *J. Neuroimmunol*. 27 (1990) 229-237.
9. Jin CY, Lee JD, Park C, Choi Y, Kim GY, Curcumin attenuates the release of pro-inflammatory cytokines in lipopolysaccharide-stimulated BV2 microglia. *Acta Pharmacol. Sin*. 28 (2007) 1645-1651.
10. Basu A, J. Krady K and Levison S. Interleukin-1: A Master Regulator of Neuroinflammation. *J Neurosci Res*. 78 (2004) 151-156.
11. Duchini A, Govindarajan S, Santucci M, Zampi G, Hofman FM, Effects of tumor necrosis factor-alpha and interleukin- 6 on fluid-phase permeability and ammonia diffusion in CNS derived endothelial cells. *J Invest Med*.44 (1996) 474-482.
12. Rock RB, Gekker G, Hu S, Sheng WS, Cheeran M, Lokensgard JR, et al. Role of Microglia in Central Nervous System Infections. *Clin Micro Rev*.17 (2004) 942-964.
13. Willis C, Leach L, Clarke GJ, Nolan CC, Ray DE. Reversible disruption of tight junction complexes in the rat blood-brain barrier, following transitory focal astrocyte loss. *GLIA*. 48 (2004) 1-13.
14. Overbergh L, Giulietti A, Valckx D, Decallonne B, Bouillon R, Mathieu C. The Use of Real-Time Reverse Transcriptase PCR for the Quantification of Cytokine Gene Expression. *J Biomol Tech*.14 (2003) 33-43.
15. Chen F, Hori T, Ohashi N, Baine AM, Eckman CB, Nguyen JH. Occludin is regulated by epidermal growth factor receptor activation in brain endothelial cells and brains of mice with acute liver failure. *Hepatology*, 53 (2011) 1294-1305.
16. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative Ct method. *Nature Protocols*, 3 (2008) 1101-1108.
17. Allan SM, Rothwell NJ. Cytokines and Acute Neurodegeneration *Nature Reviews: Neuroscience*.2 (2001) 734-744.
18. Kaushik DK, Gupta M, Das S, Basu A. Krüppel-like factor 4, a novel transcription factor regulates microglial activation and subsequent neuroinflammation *J Neuroinflamm*. 7 (2010) 1-20.
19. Doherty TM, Seder RA, Sher A. Induction and regulation of IL-15 expression in murine macrophages. *The J Immunol*.156 (1996) 735-741.
20. Giri JG, Ahdieh M, Eisenman J, Shanebeck K, Grabstein K, Kumaki S, et al. *EMBO Journal*. 13 (1994) 2822-2830.
21. Reem GH, Yeh NH. Interleukin 2 regulates expression of its receptor and synthesis of gamma interferon by human T lymphocytes. *Science*. 225 (1984) 429-430.
22. Lieb K, Engels S, Fiebich BL. Inhibition of LPS-induced iNOS and NO synthesis in primary rat

-
- microglial cells. *Neurochem Int.* 42 (2003) 131–137.
23. Block ML, Zecca L, Hong JS. Microglia-mediated neurotoxicity: uncovering the molecular mechanisms. *Nature Reviews: Neuroscience.* 8 (2007) 57-69.
 24. Conti B, Park LCH, Calingasan NY, Kim Y, Kim H, Bae Y, et al. Cultures of astrocytes and microglia express Interleukin 18. *Mol Brain Res.* 67 (1999) 46–52.
 25. Ballabh P, Braun A, Nedergaard M. The blood–brain barrier: an overview: Structure, regulation, and clinical implications. *Neurobiol Dis.* 16 (2004) 1–13.
 26. Deshmane SL, Kremlev S, Amini S, Sawaya BE. Monocyte Chemoattractant Protein-1 (MCP-1): An Overview. *J Interferon Cyto Res.* 6 (2009) 313-326.
 27. Duchini A, Govindarajan S, Santucci M, Zampi G, Hofman FM. Effects of tumor necrosis factor- α and interleukin-6 on fluid-phase permeability and ammonia diffusion in CNS derived endothelial cells. *J Invest Med.* 44 (1996) 474–482.
 28. Romano M, Sironi M, Toniatti C, Polentarutti N, Fruscella P, Ghezzi P, et al. Role of IL-6 and its soluble receptor in induction of chemokines and leukocyte recruitment. *Immunity.* 6 (1997) 315–325.
 29. Amasheh S, Meiri N, Gitter AH, Schöneberg T, Mankertz J, Schulzke JD, et al. Claudin-2 expression induces cation-selective channels in tight junctions of epithelial cells. *J Cell Sci.* 2002; 115:4969-4976.
 30. Cummins PM. Occludin: One Protein, Many Forms. *Mol. Cell. Biol.* 32 (2012) 242-250.
 31. Yang Y, Estrada EY, Thompson JF, Liu W, Rosenberg GA. Matrix metalloproteinase-mediated disruption of tight junction proteins in cerebral vessels is reversed by synthetic matrix metalloproteinase inhibitor in focal ischemia in rat. *J. Cereb. Blood Flow Metab.* 27 (2007) 697–709.
 32. Edwards DR, Beaudry PP, Laing TD, Kowal V, Leco KJ, Leco PA, et al. The roles of tissue inhibitors of metalloproteinases in tissue remodelling and cell growth. *Int. J. Obes. Relat. Metab. Disord.* 20 (1996) 9–15.

Conferences and Workshops

Conferences, Seminars and Workshops Participated

1. 1st Prize in e-paper presentation at International Symposium on Translational Research on 'Overexpression of iNOS and MMP disrupts the glial barrier in adult *Drosophila*' at First South Zone conference of Indian Academy of Biomedication Sciences organised by Department of Genetics and Genomics, University of Mysore, 8-9 September 2022.
2. Participated virtually in 'INDO-US Symposium on Molecular Virology-2022', 15-17 February 2022 sponsored by the Ministry of Education, Government of India under the Scheme for promotion of Academics and Research Collaboration (SPARC).
3. Selected for 12 days of coursework for 'Methods in *Drosophila* Biology' from 12-23 April 2021 conducted by Bangalore Life Science Cluster (BLiSC), InStem, C-CAMP, NCBS.
4. 'Research Volunteer' for (Sample aliquoting, viral RNA extraction, qRT-PCR as per WHO guidelines and result report analysis) COVID-19 Testing Team of Haffkine Institute for Training, Research and Testing, Mumbai from 24 March 2020 to till date 12.05.21, supervised by De. Shashikant Vaidya, Nodal Officer, COVID-19, HITRT Testing Team, Mumbai.
5. Participated in 'Innovation Festival 2020' held at Nehru Science Centre, 5-7 March 2020
6. Awarded 1st Prize in the 'Inter-Departmental Poster Competition on, Ideologies of Mahatma Gandhi in today's perspective' held on October 2, 2018, at the Department of Biotechnology, University of Mumbai.

7. Paper presentation and e-poster presentation at International Science Symposium on HIV & Infectious Diseases (ISSHID), from YRG Centre for AIDS Research and Sri Ramachandra Institute for Health, Education and Research on 14 October 2019.
8. Participated in ICMR-National conference on 'Upcoming Avenues for Hepatitis Elimination in India' organised by ICMR-National Institute of Immunohematology on 29 July 2018
9. Conducted 'Hands-on Training in Techniques in Virology (HOTTV-2018)' in the Department of Virology, Haffkine Institute from 4-29 June 2018.
10. Participated in 'Analytical Training for Instrumentation' at Haffkine Institute from 29-31 May 2018
11. Participated in 'Hands-on Imaging Flow Cytometry Workshop' held at National Institute for Research in Reproduction Health on 13 April 2018
12. Participated in DBT Workshop on 'Applications in Bioinformatics' sponsored by the Department of Biotechnology, Govt. of India held at Tata Memorial Centre, Advanced Centre for Treatment, Research and Education in Cancer from 8-9 March 2018.
13. Participated in 'National Science Academics Lecture Workshop' on 'Advances in Toxicology' organized by Haffkine Institute from 22-23 February 2018.
14. Participated in 'Basic Course in Molecular Biology Training Programme' conducted at Haffkine Institute from 5 July 2017 to 26 February 2018.
15. Participated in workshop on 'Nuts & Bolts of Research Methodology' organized by K. J. Somaiya College of Science & Commerce, Mumbai on 31 January 2018
16. Presented poster on the topic titled 'Essential oils: A medicated gargle against H1N1' in Virocon-2017 organised by Indian Virological Society in Nitte University, Mangalore 7-9 December 2017.

17. Attended symposium on 'Newer Diagnostic Methods in Infectious Diseases' organised by Kasturba Hospital for Infectious Diseases and Molecular Diagnostic Reference Laboratory, Mumbai on 23 June 2017.
18. Participated in a workshop on 'Principles & Practices of Laboratory Animal Care' organised by Haffkine Institute, Mumbai on 4-5 May 2017.
19. Participated in a workshop on Zotero software organised by Haffkine Institute, Mumbai on 15 March 2017.



**UNIVERSITY OF MYSORE
DEPARTMENT OF STUDIES IN GENETICS AND GENOMICS**

ORGANIZED

**FIRST SOUTH ZONE CONFERENCE OF INDIAN ACADEMY OF
BIOMEDICAL SCIENCES (IABS-SOUTH ZONE-2022)
AND
INTERNATIONAL SYMPOSIUM ON TRANSLATIONAL RESEARCH**

CERTIFICATE

THIS IS TO CERTIFY THAT PROF./DR./MR./MS.

**DURGA APPARAO BETHALA affiliated to Haffkine Institute for Training, Research and Testing, Mumbai.
has secured I / II / III / Consolation prize in oral / Poster Competition at National Conference on the
International Symposium on Translational Research, Held on 8th and 9th September, 2022 at Vijnana
Bhavan, University of Mysore, Manasagangothri, Mysuru.**

S.S. Malini

Prof. S. S. Malini
Organizing Secretary

CERTIFICATE OF ATTENDANCE

Methods in Drosophila Biology

DURGA APPARAO BETHALA

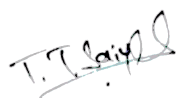
Attended all the lectures and participated in discussions/assignments/evaluation

Contents of the course:

- | | |
|---|---|
| • Basics of Drosophila Genetics | Dr. Deepti Trivedi, <i>Fly Facility, BLiSC</i> |
| • Taxonomy, Culturing, Nomenclature in Genetics, Planning Genetic Crosses | Dr. Dhananjay Chaturvedi, <i>NCBS, Bangalore</i> |
| • Studying Drosophila Development | Dr. Abhijit Das, <i>IIT Kharagpur</i> |
| • Studying nutrition in flies | Dr. Jishy Varghese, <i>IISER Trivandrum</i> |
| • Transgenesis & Generating Mutant <i>Drosophila</i> Lines | Dr. Deepti Trivedi, <i>Fly Facility, BLiSC</i> |
| • <i>Drosophila</i> as model to study Human disease | Dr. Sonal Nagarkar-Jaiswal, <i>CCMB Hyderabad</i> |
| • How to perform large scale genetic screens | Dr. Manish Jaiswal, <i>TIFR Hyderabad</i> |
| • How circadian rhythms affect fly behavior | Dr. Sheeba Vasu, <i>JNCASR, Bangalore</i> |
| • Using clonal analysis to study <i>Drosophila</i> brain | Dr. Sonia Sen, <i>TIGS Bangalore</i> |
| • Studying behavior in flies | Dr. Aman Aggarwal, <i>SciKal, Bangalore</i> |

Course Coordinator:
Dr. Deepti Trivedi

- Dates: 12th to 23rd April, 2021
- Location: Online



Dr. Taslimanif Saiyed
CEO & Director



Dr. Deepti Trivedi
In-charge, Transgenic Fly Facility



हाफकिन प्रशिक्षण, संशोधन व चाचणी संस्था

महाराष्ट्र शासन अनुदानित सोसायटीज रजिस्ट्रेशन अक्ट १८६० अधिनियमान्वये नोंदणी कृत स्वायत्त संस्था
भारत सरकार, विज्ञान व प्रौद्योगिक मंत्रालय, मान्यताप्राप्त "वैज्ञानिक आणि औद्योगिक संशोधन संस्था"

Haffkine Institute for Training, Research & Testing

An Autonomous Grant in aid Institute of Govt. of Maharashtra Registered under Societies Registration Act 1860
Recognised as "Scientific & Industrial Research Organisation" (SIRO) by Govt. of India, Ministry of Science & Technology.



HI/VYR/COVID-19 CERT/02/2020

Date: 10th August 2020

Certificate

This is to certify that **Ms. Durga Bethala** has worked as a Volunteer for the laboratory diagnosis of COVID-19 at the Department of Virology at Haffkine Institute from 24th March 2020 till date. During this period, she remained involved with Aliquoting of clinical samples, Extraction of RNA and running of PCRs. She has exhibited good laboratory skills and has worked with great enthusiasm.

We wish her all the very best for her future endeavours.

Dr. Sandeepan Mukherjee
Scientific Officer & Head
Department of Virology
Haffkine Institute

Dr. Shashikant Vaidya
Assistant Director & CAO
Haffkine Institute



पत्ता : आचार्य दोंडे मार्ग, परळ, मुंबई - ४०० ०१२. दूरध्वनी क्र. ०२२-२४१६०९४७, २४१६०९६१, २४१६०९६२, फॅक्स - ०२२-२४१६१७८७

Address : Acharya Donde Marg, Parel, Mumbai - 400 012. Tel: 022 - 24160947, 24160961, 24160962. Fax : 022 - 24161787

email : haffkineinstitute@gmail.com, info@haffkineinstitute.org, website: www.haffkineinstitute.org



नवप्रवर्तन उत्सव

INNOVATION FESTIVAL

March 5-7, **2020**

CERTIFICATE

This is to certify that

Ms. Durga Bethala

of HAFFKINE INSTITUTE FOR TRAINING, RESEARCH & TESTING

participated in the

INNOVATION FESTIVAL - 2020

held at Nehru Science Centre, Mumbai
during March 5-7, 2020

Date : 7 | 3 | 2020

Organised by:



NEHRU SCIENCE CENTRE

(National Council of Science Museums), Ministry of Culture, Govt. of India
Dr. E. Moses Road, Worli, Mumbai-400 018

Shivaprasad Khened

SHIVAPRASAD KHENED
Director
Nehru Science Centre
Mumbai

Innovations by

IITM | ACTREC | CSIR-NEERI | IDC, IITB | IIGM | NIRRH | ICAR-CIFE | CSIR-NCL
IH, NSCM | HITRT | AIF | GI | PCRA



ISSHID 2019 International Science Symposium on HIV & INFECTIOUS DISEASES 12-14, October 2019 | SRIHER, Porur, Chennai

Co-sponsored by
Department of Biotechnology, New Delhi and
SERB, Department of Science and Technology,
Government of India, New Delhi



Certificate of participation
with CME credits accredited by
Medical University / Medical Council



Academic Partners



Organized by



Certificate of Attendance

This is to certify that, **Prof. / Dr. / Mr. / Ms.**
Durga Bethala

has attended and presented (Poster) the
research abstract at the ISSHID 2019

Abstract Title:

In-silico docking of brain endothelial receptor against Dengue virus
envelope protein

Date: **12 - 14, October 2019**

Venue: **Sri Ramachandra Institute of
Higher Education and Research (SRIHER)
Chennai, India**

Prof. S. P. Thyagarajan
Organizing Chair

Dr. Sunil Suhas Solomon
Organizing Chair

Dr. Padma Srikanth
Organizing Secretary

Dr. P. Balakrishnan
Organizing Secretary



ISSHID 2019
International Science Symposium on
HIV & INFECTIOUS DISEASES
 12-14, October 2019 | SRIHER, Porur, Chennai

Co-sponsored by
 Department of Biotechnology, New Delhi and
 SERB, Department of Science and Technology,
 Government of India, New Delhi



Certificate of participation
 with CME credits accredited by
 Medical University / Medical Council



Academic Partners
 International AIDS Society (TN & PT) | BANC, Infectious Diseases | BioMed Central | SPRINGER NATURE

Organized by
 YRCARE | SRIHER

Certificate of Attendance

This is to certify that, **Prof. / Dr. / Mr. / Ms.**

Durga Bethala

**has attended and presented the
 research abstract at the ISSHID 2019**

Date: **12 - 14, October 2019**

Venue: **Sri Ramachandra Institute of
 Higher Education and Research (SRIHER)
 Chennai, India**

Prof. S. P. Thyagarajan
 Organizing Chair

Dr. Sunil Suhas Solomon
 Organizing Chair

Dr. Padma Srikanth
 Organizing Secretary

Dr. P. Balakrishnan
 Organizing Secretary



University of Mumbai

Department of Biotechnology

This is to certify that, Mr./Ms. BETHALA DURGAA has been awarded 1st Prize in the Inter-Departmental Poster Competition on "Ideologies of Mahatma Gandhi in today's perspective" held on 2nd October 2018 at the Department of Biotechnology, University of Mumbai.

Place: Mumbai

*Dr. Mrs. Archana Rath
Head, Department of Biotechnology
(Convener)*



ICMR- NATIONAL INSTITUTE OF IMMUNOHAEMATOLOGY

**NATIONAL CONFERENCE ON
"UPCOMING AVENUES FOR VIRAL HEPATITIS
ELIMINATION IN INDIA"**

Certificate of Participation

This certificate is awarded to BETHALA DURGA APPARAO
for participation/oral presentation/poster presentation at the
National Conference on Upcoming Avenues for Hepatitis Elimination in India
on 29th July 2018 at Mumbai

Dr. Aruna Shankarkumar
Organizing Secretary

Dr. Akash Shukla
Chairperson

Dr. Manisha Madkaikar
Director



हाफकिन प्रशिक्षण, संशोधन व चाचणी संस्था

महाराष्ट्र शासन अनुदानित सोसायटीज रजिस्ट्रेशन ॲक्ट १८६० अधिनियमान्वये नोंदणी कृत स्वायत्त संस्था
भारत सरकार, विज्ञान व प्रौद्योगिक मंत्रालय, मान्यताप्राप्त "वैज्ञानिक आणि औद्योगिक संशोधन संस्था"

Haffkine Institute for Training, Research & Testing

An Autonomous Grant in aid Institute of Govt. of Maharashtra Registered under Societies Registration Act 1860
Recognised as "Scientific & Industrial Research Organisation" (SIRO) by Govt. of India, Ministry of Science & Technology.



Certificate

This is to certify that **Ms. Durga Bethala** has actively participated as a resource person for the Hands on Training in Techniques in Virology (HOTTV 2018), held from 4th June 2018 until 29th June 2018. She conducted lectures and demonstrations in Animal Cell Culture and Hemagglutinin Assay during this program.

Pavankumar Todkar
Co-ordinator HOTTV 2018 &
Scientific Officer
Department of Virology
Haffkine Institute

Sandeepan Mukherjee
Co-ordinator HOTTV 2018 &
Scientific Officer
Department of Virology
Haffkine Institute

Date: 1st August 2018
Place: Mumbai

पत्ता : आचार्य दोंडे मार्ग, परळ, मुंबई - ४०० ०१२. दूरध्वनी क्र. ०२२-२४१६०९४७, २४१६०९६१, २४१६०९६२, फॅक्स - ०२२-२४१६१७८७

Address : Acharya Donde Marg, Parel, Mumbai - 400 012. Tel: 022 - 24160947, 24160961, 24160962. Fax : 022 - 24161787

email : haffkineinstitute@gmail.com, info@haffkineinstitute.org, website: www.haffkineinstitute.org



Haffkine Institute

for Training, Research & Testing

Autonomous Institute of Government of Maharashtra, An ISO 9001:2008 Certified Organisation.
Recognized as "Scientific & Technical Research Organisation" (STRO) by Ministry of Science & Technology, Govt. of India.

Certificate

awarded to

Dr./Mr./Ms. BETHALA DURGA APPARAO

for actively participating in ANALYTICAL TRAINING FOR INSTRUMENTATION

conducted from 29/05/18 to 31/05/18 under the aegis of

Haffkine Institute for Training, Research & Testing, Mumbai.

Evans Coordinator
Haffkine Institute, Mumbai

Director
Haffkine Institute, Mumbai

HI/CYR/AT/25/2018

IMAGING FLOW CYTOMETRY WORKSHOP



Power of Microscope with High speed analysis of Flow Cytometry

Certificate of Participation

*This is to certify thatMr. Durga Bhatla..... of
Haffkine Institute...for training, research... has successfully participated in the
Hands-on "Imaging Flow Cytometry Workshop" held at the National
Institute For Research In Reproductive Health on April 13, 2018.*

V. V. Patel

Dr. Vainav Patel
Head, Dept. of Biochemistry &
Virology, NIRRH (ICMR)

Sagar U. Shah

Mr. Sagar U. Shah
Application Specialist,
Imperial Life Sciences (P) Ltd



DBT Workshop on
'Applications in Bioinformatics'

March 8th - 9th, 2018

This is to certify that

Durga Bethala

participated in the workshop
sponsored by the Dept. of Biotechnology (DBT), Govt. of India,
held at Tata Memorial Centre,
Advanced Centre for Treatment, Research and Education in Cancer (ACTREC),
Kharghar, Navi Mumbai - 410 210.

S. Chiplunkar

Dr. Shubhada Chiplunkar
Director, ACTREC

A. Varma

Dr. Ashok Varma
Co-ordinator
Bioinformatics Centre
ACTREC





Haffkine Institute

for Training, Research & Testing

Autonomous Institute of Government of Maharashtra, An ISO 9001:2008 Certified Organisation.
Recognized as Scientific & Industrial Research Organisation (SIRO) by Ministry of Science & Technology, Govt. of India.

Certificate

awarded to

Dr./Mr./Ms. Pooja Belhale

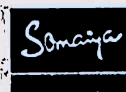
for actively participating in *Molecular Biology Training Program (Basic Course)*
conducted from 5th July 2017 to 26th February 2018.

Director
HITRI, Mumbai



K. J. Somaiya College of Science and Commerce

Autonomous College - Affiliated to University Of Mumbai
Reaccredited 'A' Grade by NAAC with CGPA 3.24
College of Potential for Excellence (2016)



CERTIFICATE

This is to certify that Bys. / Sis. Durga Apparao Bethala of Hoffkine Institute for Training, Research and Testing college has participated in a One day workshop on 'Nuts and Bolts of Research Methodology' organised by IQAC held on 31st January, 2018.


Dr. Pradnya Prabhu
Vice Principal
&
IQAC Coordinator


Dr. Vilas Joshi
Principal

NITTE

Indian Virological Society & Nitte University



VIROCON 2017

**26th Annual Conference of Indian Virological Society (IVS)
"Viruses to Viromes in Health and Disease"**

Participation Certificate

This is to certify that Prof./Dr./Mr./Ms. Bethala Durga Apparao has participated and presented oral / poster at the 26th Annual conference **"VIROCON - 2017"** during 7 - 9 December organised by Nitte University, Deralakatte, Mangaluru, India.


Prof. Anupam Varma
President
Indian Virological Society


Dr. G.P. Rao
General Secretary
Indian Virological Society


Prof. Iddya Karunasagar
Chairman
Organising Committee



**KASTURBA HOSPITAL FOR INFECTIOUS DISEASES &
MOLECULAR DIAGNOSTICS REFERENCE LABORATORY**

SYMPOSIUM

'NEWER DIAGNOSTIC METHODS IN INFECTIOUS DISEASES'

Certificate

This is to certify that..... Bethala Durga Apparao
was a Faculty/Delegate in the Symposium on 'Newer Diagnostic Methods in Infectious
Diseases' organised by Kasturba Hospital for Infectious Diseases, Mumbai on
Friday, 23rd June 2011.

Maharashtra Medical Council has granted Two/Three credit hours.

Dr. Jayanthi Shastri
Professor & Head, Microbiology, TNMC
Incharge, Molecular Laboratory
Kasturba Hospital, Mumbai.

Dr. Chandrakant Pawar
Medical Superintendent
Kasturba Hospital, Mumbai

Observer
Maharashtra Medical Council

PPLAC_13-CT)-04



Haffkine Institute

for Training, Research & Testing

Autonomous Institute of Government of Maharashtra, An ISO 9001:2008 Certified Organisation.
Recognized as "Scientific & Industrial Research Organisation" (SIRO) by Ministry of Science & Technology, Govt. of India.

Certificate

awarded to

Dr./Mr./Ms. BETHALA DURGA APPARAO

for actively participating in Workshop On "PRINCIPLE AND PRACTICES OF LABORATORY ANIMAL CARE"

conducted from 4/5/2017 to 5/5/2017 under the aegis of

Haffkine Institute for Training, Research & Testing, Mumbai.

Since 1899

Havant
Course Coordinator
Haffkine Institute, Mumbai

Mr. Mah
Director
Haffkine Institute, Mumbai



Haffkine Institute

for Training, Research & Testing

Autonomous Institute of Government of Maharashtra, An ISO 9001:2008 Certified Organisation.
Recognized as "Scientific & Industrial Research Organisation" (SIRO) by Ministry of Science & Technology, Govt. of India.

Certificate

awarded to

Dr./Mr./Ms. Durga Bethala

for actively participating in Workshop on Zetox

conducted from 15/03/2017 to - under the aegis of

Haffkine Institute for Training, Research & Testing, Mumbai.

[Signature]
Course Coordinator
Haffkine Institute, Mumbai

[Signature]
CHAIRPERSON
LIBRARY COMMITTEE
HAFFKINE INSTITUTE, PAREL, MUMBAI

[Signature]
Director
Haffkine Institute, Mumbai

**SYNOPSIS SUBMISSION
TO THE UNIVERSITY OF MUMBAI
FOR THE DEGREE OF DOCTOR PHILOSOPHY (SCIENCE) IN
APPLIED BIOLOGY**

TITLE OF THE THESIS: IDENTIFICATION OF LIGAND-RECEPTOR
INTERACTION ACROSS BLOOD BRAIN BARRIER
(BBB) IN VIRAL NEURO-PATHOGENESIS

NAME OF THE
CANDIDATE: Ms. Bethala Durga Apparao

BASIC QUALIFICATION
OF THE CANDIDATE: M.S. (Pharm) Biotechnology

NAME AND
DESIGNATION OF THE
GUIDE: Dr. Shashikant Prabhakar Vaidya
Assistant Director & Head, Department of Clinical
Pathology

PLACE OF RESEARCH
WORK: Department of Clinical Pathology, Haffkine Institute for
Training, Research and Testing, Acharya Donde Marg,
Parel, Mumbai-400012.

REGISTRATION NO. &
DATE: 61/ 26.06.2019

SIGNATURE OF THE
CANDIDATE:

SIGNATURE OF THE
GUIDE:

Title of the project:

IDENTIFICATION OF LIGAND-RECEPTOR INTERACTION ACROSS BLOOD BRAIN BARRIER (BBB) IN VIRAL NEURO-PATHOGENESIS

Funding Source: DST-INSPIRE Fellowship, Department of Science and Technology, Government of India.

Abstract:

More than 500 million people worldwide are infected each year by any of the four-dengue virus (DENV) serotypes. Dengue serotype 2 (DENV 2) is most predominant in India and other South Asian countries. The clinical spectrum caused during dengue infections is wide and some patients may develop neurological alterations during or after the infection. Clinical evidence suggests that blood brain barrier (BBB) may be compromised during DENV infection; however, it is not clear whether the damage is due to the infection per se or to the inflammatory response activated by the BBB cells. Other neurotropic flaviviruses such as Japanese encephalitis virus (JEV) are also known for neurological damage. JEV uses the bloodstream to enter nerve tissue where it infects cells of the neurovascular unit. Another neurotropic virus like rabies virus (RABV) chooses the retro-grade axonal route to infect the neuronal cells. Both JEV and RABV affect the cells of neurovascular unit in different ways resulting into BBB damage, glial activation, and tissue inflammation. Our aim is to study pattern of DENV infection to BBB, compared with other neurotropic viruses like JEV and RABV using *in vitro* model. This information will allow us to understand impact of dengue virus on the complex nervous system.

1. Introduction:

In the past two decades, dengue transmitted by *Aedes aegypti* and *Aedes Albopictus* mosquito species has appeared as the most commonly occurring arthropod-borne flavivirus illness worldwide. From a general picture of its epidemiology, it is estimated that approximately 3-5 billion infections occur each year, of which nearly a quarter is characterized with symptoms. Following infection, dengue disease manifests as an array of clinical signs that varies from a non-specific febrile illness, known as dengue fever (DF), to life-threatening forms, addressed as dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS) found mostly in tropical and sub-tropical climate. Another flavivirus, Japanese encephalitis virus (JEV) is one of the most important causes of viral encephalitis worldwide, with no specific antiviral treatment available. Despite some recent successes with widespread vaccination, JEV a neurotropic virus will likely remain an important public health problem; because the virus is mosquito-borne and has natural animal hosts, it will never be eradicated. We have little understanding of what determines the severity and outcome of infection. Circumstantial evidence suggests that in addition to cytopathology caused directly by infection of neurons, there may be bystander cell death of non-infected neurons, caused by an excessive inflammatory response. Another example of neurotropic viruses, the RABV is an ancient and fatal neurological disease of animals and humans, caused by infection of the central nervous system (CNS) belongs to the family *Rhabdoviridae*. It is estimated that nearly 55,000 human RABV fatalities occur each year, though this number is likely much higher due to unreported exposures or failure of diagnosis. No treatment has been identified to cure disease after onset of symptoms. Experts still do not know the cause of rabies dramatic symptoms and fatality, though it is thought to be due to neuronal loss or dysfunction.

The frequency of neurological manifestations upon flavivirus infection varies considerably depending on the virus. Even though these viruses usually do not affect the CNS, a recent surveillance study performed in Brazil reported the presence of laboratory markers of DENV and zika virus (ZIKV) in patients with acute neurological diseases. Among 74 studied cases, 2 patients presented DENV RNA in the cerebrospinal fluid (CSF) and 6 patients showed anti-DENV immunoglobulins in the serum. These data suggest that arthropod borne viruses may play a more important role in CNS-associated disease than previously found, at least in endemic countries where the number of infected individuals is very high. All the referred viruses can infect neurons, though they are not always neuroinvasive. Viruses can reach the brain through

different mechanisms, including peripheral nervous system and axonal transport and hematogenous route. Although experimental models had previously indicated that some flaviviruses, such as west nile virus (WNV) and JEV, spread via axonal transport or through the olfactory bulb, those models were developed using intra-sciatic and intranasal inoculation, which may not represent their natural infection route. Given the systemic nature of flavivirus infection and since a disruption of the BBB is often observed in experimental models of infection, it is believed that crossing this structure is a major mechanism for flavivirus. Dengue is considered an important neglected tropical disease, wherein World Health Organisation (WHO) has included the neurological manifestation as a clinical presentation in DHF or in severe Dengue cases like in DSS. Neurological manifestations of DENV may occur during or after acute infection, it may be transient or irreversible. The proposed investigation is a comparative study on BBB infection between DENV and other neurotropic virus such JEV and RABV. This study on brain endothelial cells could greatly add to our understanding of the neurotropism and neuropathogenesis associated with the DENV.

2. Objectives:

- 5) To evaluate the neurotoxicity of DENV, JEV, RABV viruses on cerebral microvascular endothelial cells.
- 6) To study effect of inflammatory cytokine on DENV, JEV, RABV virus infection on endothelial cells
- 7) To study the effect of DENV, JEV, RABV virus on endothelial cells tight junction protein
- 8) To evaluate the effect of DENV, JEV, RABV virus infection on MMP-2 and MMP-9 matrix metalloproteinases

3. Methodology:

3.1 Propagation of cell lines:

For the propagation of the virus and to study the effect of virus on BBB. The bEnd-3, PS, N2a, Vero and BHK 21 cell lines were propagated as per the American Type Culture Collection (ATCC) & National Centre for Cell Science (NCCS) protocol and were stored in vapour phase of liquid nitrogen.

Cell Line	Type	Source	Purpose
bEnd3	Brain endothelial	Mouse	Identify receptor protein involved in virus neuroinvasion
N2a	Neuroblast	Mouse	Neuronal response
SHSY5Y	Neuroblast	Human	Neuronal response
PS	Epithelial	Porcine	JEV propagation
BHK21	Kidney Fibroblast	Hamster	DENV and RABV propagation
Vero	Fibroblast	Monkey	JEV propagation

3.2 Propagation of Viruses:

DENV-2 strain were propagated into BHK21 cell line while JEV GP-78 strain were propagated into Porcine Stable (PS) & Vero cell line. Rabies virus Challenge Virus Standard (CVS-11) strain were propagated into BHK-21 cell line. The CVS-11 were procured from Central Research Institute (CRI), Kasauli was used for the study. The virus was passaged onto the BHK-21 cell line so that the viruses adapt themselves to the cell line and recognize them while doing the infections during the main assays. For preparing the virus stocks, the viruses were made to infect the confluent monolayer of low passage number BHK-21 (Passage Number# 21)

3.3 Primary- Direct Infection with DENV on BBB endothelial cells:

Brain Endothelial-3 cell line (bEnd3) was used as substrate for BBB endothelial cells model which were procured from ATCC-USA. This contained 7.5% Sodium bicarbonate, and 10% Fetal Bovine Serum (FBS). Cells were maintained at 37°C under 5% CO₂ till confluence was

reached. They were sub-cultured using mechanical disruption procedures/scraping and centrifuged at 1500rpm for 10 minutes. They were split in the ratio of 1:2 to 1:4 as per requirement. bEnd3 cells were seeded in a 25 cm² flask and incubated overnight in Dulbecco Medium Essential Media (DMEM) with 2% FBS. Cells were primary infected with the Japanese encephalitis virus, Dengue virus and Rabies virus at multiplicity of infection 0.1 (m.o.i = 0.1). This infection was carried out for 2 hours with JEV GP-78, DENV2 and CVS-11 respectively in different flasks and in triplicate. The virus infection on bEnd3 cells were evaluated by quantitative real time polymerase chain reaction (qRT-PCR) using SYBR-GREEN chemistry. While some of the supernatant was collected from the virus infected bEnd3 wells for protein analysis and the cells were processed for extraction of transmembrane protein.

3.4 Infection of virus activated monocytes on BBB endothelial cells and Neuronal cells:

Murine monocyte (RAW) cell line was used as model for stimulus of immune response. Firstly, the RAW cells were infected with JEV GP-78, DENV2 and CVS-11 (moi = 0.1). This infection was carried out for 2 hours with JEV GP-78, DENV2 and CVS-11 respectively in different flasks and in triplicate. Post-infection the virus activated RAW cells supernatant was collected and it was over-layed on bEnd3 cells and on neuronal cells like SH-SY-5Y and N2a cell lines. The later infection was carried out with virus activated monocyte supernatant after 2 hours of primary infection, incubating for further 2 hours. An identical set of control flasks were also propagated. The virus infection on RAW-bEnd3 cells were evaluated by qRT-PCR using SYBR-GREEN chemistry. Further, the bEnd3 and neuronal cells supernatant was collected for protein analysis and the cells were processed for extraction of transmembrane protein.

3.5 Estimation of cytotoxicity:

Cytotoxicity due to JEV GP-78, DENV2 and CVS-11 primary and virus-exposed monocyte supernatants on bEnd3 was assessed using the 3-(4,5-Dimethylthiazol-2-YI)-2,5-Diphenyltetrazolium Bromide (MTT) assay with minor modifications. Briefly, each virus was inoculated at an moi of 0.01 to study the cell viability in 96 well plates of RAW, Bend 3, SH-SY-5Y and N2a cells. Identical plates were also maintained for DENV2 infection, while 50 dimethyl sulphoxide (DMSO) was used as a negative control. After incubation at 37°C with 5% CO₂ for 16-18 hours, 10% of 5mg/ml MTT (100µl) was added to each well. After 4 hours

of incubation at 37°C, the formazan was solubilized by adding DMSO to each well and the absorbance was read at 550nm by a multimode reader.

3.6 Standardization of protein-protein interaction:

Using PATCHDOCK & FIREDOCK program the potential receptor protein noted for JEV and Rabies virus is being screened for Dengue envelope protein. The structure with lowest binding energy score was selected to identify important amino acids at the interface region.

3.7 HBEC-5i Membrane protein extraction:

The transmembrane protein of non-infected HBEC 5-i and virus infected HBEC-5i cells were extracted using FOCUS Membrane Proteins kit (Cat. #786-249) protocol. The aliquoted interphase comprising hydrophobic membrane protein fraction was stored at –80°C until used. Total protein concentration was determined by Bradford assay.

3.8 Transmembrane protein determination by Bradford Assay:

Bovine serum albumin (BSA) as standard protein at concentration range of 20-300µg/µl protein. The virus infected bEnd3 cells (test) and non-infected bEnd3 (control) were used as neat, and absorbance was measured at 595 nm using the multimode plate reader.

3.9 Viral RNA Extraction:

The respective viral RNA was extracted from cells culture supernatant harvested after successful virus propagation. Viral RNA isolation kit was used for the RNA extraction of DENV2, JEV GP-78, CVS-11 viruses from infected cells. Briefly, 10^6 - 10^8 cells were subjected to lysis and extraction and elution steps. The extracted RNA was reverse transcribed to cDNA using PCR thermal cycler and stored at -80°C until further analysis was carried out for studying cytokine gene expression.

3.10 Gene expression analysis:

Gene expression was categorized into four sets (i) Inflammatory: Interleukin-1 beta (IL-1), Tumor Necrosis Factor alpha (TNF-α), Interferon gamma (IFN-γ) and Interleukin-18 (IL-18) (ii)Regulatory: Monocyte chemoattractant protein-1 (MCP-1) and Interleukin-6 (IL-6) (iii) BBB breakdown: Occludin, Matrix metalloproteinases (MMP2, MMP9) and Tissue Inhibitor

of Metalloproteinase (TIMP1) and (iv) Reactive Oxygen Species: Inducible nitric oxide synthase (iNOS). Amplification was carried out using qRT-PCR kit. The primers and cycling conditions were used as described elsewhere (Overberghet al., 2003; Chen et al., 2011), while GAPDH was employed as the housekeeping gene. Three separate cells flasks were utilized to calculate ΔCt value for each gene. Fold change was calculated using the $\Delta\Delta\text{Ct}$ value determination, using mean ΔCt value. Change was termed as significant when $\Delta\Delta\text{Ct}$ value was more than 2 as per Schmittgen and Livak, 2008 method.

4. Findings:

4.1 Cytotoxicity analysis of virus infection on brain endothelial cells:

- The JEV GP-78 and DENV2 were more susceptible to the bEnd3 cells as compared to the other neuronal cells viz. SHSY5Y and N2a cells while Rabies has null effect on bEnd 3 cell line which shows that RABV doesn't have much affinity towards murine endothelial cells.
- The analysis of direct infection of DENV2, JEV GP-78 and CVS-11(rabies virus) shows us that DENV2 is more cytotoxic to the bEnd 3 cells as compared to the other two neurotropic viruses
- When we infect the monocytic RAW cells with this three different viruses and post 4 hours infection we collect the supernatant and overlay on the bEnd3 cells to see its effect on cell viability.
- Upon 2 hours incubation with the supernatant, we observe that DENV2 infected supernatant is more cytotoxic than the JEV GP-78 and RABV directly infected.

4.2 *Insilico* brain endothelial cell receptor identification against Dengue envelope protein:

- We used SWISSMODEL software to create a stable 3D structure of Dengue envelope protein having three important domain (D) D I, D II, D III of which we found the DIII is highly involved in receptor binding entry mechanism.
- We used PATCHDOCK and FIREDOCK protein-protein docking software to identify the host brain endothelial cell receptors binding to the Dengue envelope protein

- We screened the receptors identified in different flavivirus receptor mediated entry mechanism and the receptor involved in rabies infection from the literature.
- Annexin 6, PLVAP and VLP13-C are the three-receptor found to be having lesser binding energy than its earlier identified virus partner.

4.3 Analysis of transmembrane protein expression post different virus infection:

- Upon direct infection of DENV2, JEV GP-78, CVS-11 we see an alleviated transmembrane protein expression in JEV and DENV infection suggesting the infection susceptibility of flavivirus on brain endothelial cells.
- A lower expression of transmembrane protein was observed when bEnd3 cells were incubated with supernatants of DENV2 infected RAW cells suggesting an downregulation of tight junction protein maybe due to cytokine infiltration.

4.4 Post infection gene expression analysis:

- We see an alleviated iNOS expression in dengue direct and dengue activated monocyte supernatants suggesting the dengue virus induced oxidative stress on endothelial cells.
- At the same time we also observe an upregulation in the MMP2 expression and downregulation of Occludin tight junction protein suggesting an oxidative stress induced activation of proteolytic disruption of BBB during dengue virus infection.

Though iNOS gene expression is increased in primary infections with JEV GP-78 and DENV2, further induction is lost in infection with CVS-11. DENV2 super-infection appears to boost the oxidative stress in bEnd3 primary infection, as seen by an extensive upregulation of iNOS, in turn damaging the basal lamina of the BBB. This again aids in the infiltration of cells into the CNS. Most severe impairment of the BBB is seen in DENV2 activated RAW on bEnd3, wherein Occludin expression falls, juxtaposed with an increment in the mRNA levels of MMP-2, a moiety reported to cleave Occludin and loosen the tight junctions.

On a composite view, it is apparent that though iNOS and MMP2 aids in the impairment of the BBB, their chief role is to recruit the peripheral monocytes towards the site of infection. Our study is a novel work illustrating the scenario at the CNS barrier during DENV2 infection.

Further studies with such directed approach would aid in alleviating the perception towards the infection of DENV2 in the brain.

The Thesis is divided into the following chapters:

Chapter I: Gives a Brief **Introduction** to the Topic.

Chapter II: Includes **Review of Literature** wherein up to date information is given on the related area of research.

Chapter III: Elaborates the **Materials and Methods** used in carrying out this research work along with statistical tests used.

Chapter IV: Deals with the **Results** of various experiments which includes Tables, Figs and statistical calculations.

Chapter V: It consist of **Discussion** wherein the results are interpreted and valid conclusions are drawn from the data obtained and existing literature.

Chapter VI: Gives the **Summary** of the research work.

Chapter VII: The literature cited in the text is presented in alphabetical order as **Bibliography**.

References:

Aspelund, A. et al. A dural lymphatic vascular system that drains brain interstitial fluid and macromolecules. *J. Exp. Med.* 212, 991–999 (2015).

Daniels, B. P. & Klein, R. S. Knocking on closed doors: host interferons dynamically regulate blood-brain barrier function during viral infections of the central nervous system. *PLoS Pathog.* 11, e1005096 (2015).

Daniels, B. P. et al. Viral pathogen-associated molecular patterns regulate blood-brain barrier integrity via competing innate cytokine signals. *mBio* 5, e01476-14 (2014).

Hagendorf, N. & Conzelmann, K.-K. Recombinant fluorescent rabies virus vectors for tracing neurons and synaptic connections. *Cold Spring Harb. Protoc.* <http://dx.doi.org/10.1101/pdb.top089391> (2015).

Hagendorf, N. & Conzelmann, K.-K. Recombinant fluorescent rabies virus vectors for tracing neurons and synaptic connections. *Cold Spring Harb. Protoc.* <http://dx.doi.org/10.1101/pdb.top089391> (2015).

Iwasaki, Y., Ohtani, S. & Clark, H. F. Maturation of rabies virus by budding from neuronal cell membrane in suckling mouse brain. *J. Virol.* 15, 1020–1023 (1975).

Koyuncu, O. O., Hogue, I. B. & Enquist, L. W. Virus infections in the nervous system. *Cell Host Microbe* 13, 379–393 (2013).

Libbey, J. & Fujinami, R. S. Adaptive immune response to viral infections in the central nervous system. *Handb. Clin. Neurol.* 123, 225–247 (2014).

Lobigs, M., Mullbacher, A., Wang, Y., Pavy, M. & Lee, E. Role of type I and type II interferon responses in recovery from infection with an encephalitic flavivirus. *J. Gen. Virol.* 84, 567–572 (2003).

McGavern, D. B. & Kang, S. S. Illuminating viral infections in the nervous system. *Nature* 11, 318–329 (2011).

Van den Pol, A. N., Ding, S. & Robek, M. D. Long distance interferon signaling within the brain blocks virus spread. *J. Virol.* 88, 3695–3704 (2014).

Wakim, L. M., Woodward-Davis, A. & Bevan, M. J. Memory T cells persisting within the brain after local infection show functional adaptations to their tissue of residence. *Proc. Natl Acad. Sci. USA* 107, 17872–17879 (2010).

Wang, T. et al. Toll-like receptor 3 mediates West Nile virus entry into the brain causing lethal encephalitis. *Nat. Med.* 10, 1366–1373 (2004).

Zaichick, S. V. et al. The herpesvirus VP1/2 Protein is an effector of dynein-mediated capsid transport and neuroinvasion. *Cell Host Microbe* 13, 193–203 (2013) Podolsky, M. A. et al.



हाफकिन प्रशिक्षण, संशोधन व चाचणी संस्था

महाराष्ट्र शासन अनुदानित सोसायटीज रजिस्ट्रेशन अंक्ट १८६० अधिनियमान्वये नोंदणी कृत स्वायत्त संस्था
भारत सरकार, विज्ञान व प्रौद्योगिक मंत्रालय, मान्यताप्राप्त "वैज्ञानिक आणि औद्योगिक संशोधन संस्था"

Haffkine Institute for Training, Research & Testing

An Autonomous Institute of Govt. of Maharashtra Registered under Societies Registration Act 1860

Recognised as "Scientific & Industrial Research Organisation" (SIRO) by Govt. of India, Ministry of Science & Technology.



MEMBERS

Dr. Tarala Nandedkar
Chairperson, IEC, Haffkine Institute

Dr. Shobhna Bhatia
Prof. & Head - Gastroenterology
Seth G S Medical College & KEM Hospital
Mumbai

Dr. Sadhana Sathaye
Prof. of Pharmacy, ICT
Mumbai

Dr. Lalita Savardekar
Scientist E - Dept. of Clinical Research,
NIRRH, Mumbai

Dr. Swati Bal-Tembe
VP (Retd.) - Corporate Communications,
Piramal Enterprises Ltd.
Advocate, Bar Council of India

Ms. Amruta Lovekar
Consultant Trainer
Regional Resource Training Centre
of the National Institute of Social Defense

Ms. Shubhada Chaukar
Journalist

Dr. Anuya Rege
Head - Dept. of Bacteriology
Haffkine Institute

Dr. Sandesha Pashte
Medical Officer
Haffkine Institute

Dr. Sweta T Kothari
Head - Dept. of Virology &
Member Secretary - IEC
Haffkine Institute

Mr. Virendra Yagnik
Philosopher

HITRT/IEC/Exemption/2017/02

Date: 28/6/18

Institutional Ethics Committee (IEC) Approval

Exemption Certificate

This is to certify that the project titled "Identification of ligand-receptor interaction across BBB in viral neuropathogenesis" proposed by Dr. Sweta T Kothari has been reviewed by the IEC, Haffkine Institute for Training, Research and Testing, Parel, Mumbai - 400012, in the meeting held on 26th October 2017.

IEC has exempted this project as there is no human sample involvement.

J N
Dr. Tarala Nandedkar
Chairperson, IEC
Haffkine Institute

पत्ता : आचार्य दोंडे मार्ग, परळ, मुंबई - ४०० ०१२. दूरध्वनी क्र. ०२२-२४१६०९४७, २४१६०९६१, २४१६०९६२, फॅक्स - ०२२-२४१६१७८७

Address : Acharya Donde Marg, Parel, Mumbai - 400 012. Tel: 022 - 24160947, 24160961, 24160962. Fax : 022 - 24161787

email : haffkineinstitute@gmail.com, website: www.haffkineinstitute.org



हाफकिन प्रशिक्षण, संशोधन व चाचणी संस्था

महाराष्ट्र शासन अनुदानित सोसायटीज रजिस्ट्रेशन अंक्ट १८६० अधिनियमान्वये नोंदणी कृत स्वायत्त संस्था
भारत सरकार, विज्ञान व प्रौद्योगिक मंत्रालय, मान्यताप्राप्त "वैज्ञानिक आणि औद्योगिक संशोधन संस्था"

Haffkine Institute for Training, Research & Testing

An Autonomous Institute of Govt. of Maharashtra Registered under Societies Registration Act 1860

Recognised as "Scientific & Industrial Research Organisation" (SIRO) by Govt. of India, Ministry of Science & Technology.



MEMBERS

Dr. Nishigandha Naik

Director;
Head, Dept. of Toxicology & Biochemistry
Chairperson, IBSC

Dr. Atmaram Bandivadekar

Consultant, NIRRH, Mumbai
Invited External Expert Member, IBSC

Dr. Sweta T Kothari

Head, Dept. of Virology
Biosafety Officer, IBSC

Dr. Shital Mane

Medical Officer, Dispensary

Dr. Usha Padmanabhan

Head, Dept. of Cell Biology

Ms. Amruta Naik

SO, Dept. of Biochemistry
Member Secretary - IBSC

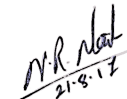
Institutional Bio-Safety Committee (IBSC)

Ref:
HITRT/IBSC/2017/C-2

21st August, 2017

Project Approval Certificate

This is to certify that the project titled "Identification of ligand-receptor interaction across BBB in viral neuro-pathogenesis" proposed by **Dr. Sweta T Kothari** has been approved by the IBSC of the, Haffkine Institute for Training, Research and Testing, Parel, Mumbai 400012, in the meeting held on 18th August 2017.


21.8.17
Dr. Nishigandha Naik
Chairperson, IBSC

पत्ता : आचार्य दोंडे मार्ग, परळ, मुंबई - ४०० ०१२. दूरध्वनी क्र. ०२२-२४१६०९४७, २४१६०९६१, २४१६०९६२, फॅक्स - ०२२-२४१६१७८७

Address : Acharya Donde Marg, Parel, Mumbai - 400 012. Tel: 022 - 24160947, 24160961, 24160962. Fax : 022 - 24161787

email : haffkineinstitute@gmail.com, website: www.haffkineinstitute.org

