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# **Development of a Novel Delivery System for Post- Infection Treatment of Rabies**

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St. Georges, University of London

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Thesis submitted for the degree of Doctor  
of Philosophy



## **Declaration of Authorship**

I, Samuel Peter Smith, declare that this thesis, and the work presented in it, is entirely my own.  
Where I have consulted the work of others, this has been clearly stated.

Samuel Peter Smith

A handwritten signature in black ink, appearing to read 'SP Smith', with a long horizontal flourish extending to the right.

# Thesis Summary

Rabies virus (RABV) causes possibly the oldest and most feared disease of mankind and is responsible for >59,000 human fatalities/year. Post exposure prophylaxis (PEP), the administration of vaccine and rabies immunoglobulin, is a highly effective tool but largely unavailable in areas where RABV is endemic. Furthermore, due to the constraints of the Blood-Brain Barrier, current PEP regimes are ineffective after the onset of clinical symptoms, which invariably results in death.

To circumvent this barrier, this thesis focuses on the design of a live attenuated recombinant RABV expressing a highly RABV-neutralising scFv antibody (62-71-3). 62-71-3 is a highly characterised mAb directed against antigenic site I (AS-I) of the RABV G protein. 62-71-3 has been shown to effectively neutralise RABV when expressed as either a full-length IgG or scFv. Using reverse genetics, 62-71-3 scFv was inserted at the front of the RABV genome for optimal expression and linked to the fluorophore mCHERRY for visualisation under UV illumination. Furthermore, to avoid self-neutralisation, AS-I was mutated to diminish 62-71-3 scFv binding. Once rescued, the resulting construct (named RABV-62scFv) was grown to high titres and its growth and cellular dissemination kinetics characterised. In addition, binding and neutralisation studies demonstrated that functional 62-71-3 scFv was effectively secreted in neutralising concentrations in cell culture.

RABV-62scFv was then taken forward to *in vivo* studies in mice where it was shown to be non-lethal when administered at high doses ( $10^4$  FFU) intracranially. Intracranial administration was also shown to elicit high serum neutralisation titres. To assess whether RABV-62scFv treatment was able to prevent mortality, mice were treated with an intracranial injection of RABV-62scFv either concurrently, three, or five days after lethal RABV (RV437) challenge. This revealed that intracranial RABV-62scFv treatment significantly increased survival rate (33% survival when given 3 dpi) and delayed the progression of symptoms.

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First and foremost, I would like to dedicate this thesis to my late father, Pete. Despite writing an entire thesis, I struggle with finding the words for this paragraph. You have been there throughout my entire life, seen me at both my lowest points in my PhD and the highest. I know that you didn't have a clue what I was talking about most of the time, but I couldn't have done it without you.

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# Table of Contents

|                                                                                   |    |
|-----------------------------------------------------------------------------------|----|
| Thesis Summary .....                                                              | 3  |
| Acknowledgements.....                                                             | 4  |
| Table of Contents.....                                                            | 6  |
| List of Figures .....                                                             | 11 |
| List of Tables .....                                                              | 15 |
| Abbreviations.....                                                                | 17 |
| Chapter 1 - Introduction .....                                                    | 27 |
| 1.1 Rabies Virus .....                                                            | 27 |
| 1.1.1 The structure and shape of the RABV.....                                    | 27 |
| 1.2 Proteins of RABV.....                                                         | 28 |
| 1.2.1 N protein.....                                                              | 28 |
| 1.2.2 P protein .....                                                             | 29 |
| 1.2.3 M protein.....                                                              | 30 |
| 1.2.4 G protein.....                                                              | 31 |
| 1.2.4.1 Cell membrane fusion activities of the RABV G. ....                       | 31 |
| 1.2.4.2 Receptors for viral infection.....                                        | 32 |
| 1.2.5 L Protein .....                                                             | 33 |
| 1.3 Life Cycle of RABV .....                                                      | 34 |
| 1.4 Structure of the brain and nervous system.....                                | 35 |
| 1.4.1 The blood-brain barrier and neurovascular unit .....                        | 36 |
| 1.4.2 Viral dissemination within the host and the onset of clinical disease ..... | 39 |
| 1.4.3 Symptoms of RABV infection.....                                             | 39 |
| 1.5 Immune response to RABV infection.....                                        | 40 |
| 1.5.1 Innate Immune response to RABV infection .....                              | 41 |
| 1.5.1.1 Pattern recognition receptors of the innate immune system .....           | 42 |
| 1.5.1.2 Innate immunity in the brain .....                                        | 43 |
| 1.5.2 The adaptive immune response to RABV infection.....                         | 44 |
| 1.5.2.1 Importance of humoral immunity in RABV clearance from the CNS. ....       | 45 |
| 1.5.3 Immune evasion .....                                                        | 46 |
| 1.5.3.1 N.....                                                                    | 47 |
| 1.5.3.2 P.....                                                                    | 48 |
| 1.5.3.3 M .....                                                                   | 48 |
| 1.5.3.4 G .....                                                                   | 48 |
| 1.5.3.5 L.....                                                                    | 49 |
| 1.6 Pre- and Post-exposure Rabies management .....                                | 49 |

|                                                                                  |    |
|----------------------------------------------------------------------------------|----|
| 1.6.1 Pre-exposure prophylaxis .....                                             | 50 |
| 1.6.1.1 PrEP Vaccines - Current research into live viruses and recombinants..... | 51 |
| 1.6.1.1.1 Characteristics of attenuated viruses.....                             | 52 |
| 1.6.1.1.2 Methods of RABV attenuation .....                                      | 52 |
| 1.6.1.1.2.1 Attenuation by Mutation.....                                         | 52 |
| 1.6.1.1.2.2 Insertion of non-viral elements.....                                 | 54 |
| 1.6.2 Post-exposure treatments and prophylaxis .....                             | 56 |
| 1.6.2.1 Milwaukee protocol .....                                                 | 56 |
| 1.6.2.2 Monoclonal antibodies .....                                              | 56 |
| 1.6.2.2.1 62-71-3 .....                                                          | 56 |
| 1.6.2.2.2 The successful treatment of clinical rabies in mice.....               | 58 |
| 1.6.3 Concluding statements .....                                                | 58 |
| 1.7 Project Outline.....                                                         | 60 |
| Chapter 2 - Methods and Materials.....                                           | 61 |
| 2.1 Standard Buffers and Solutions .....                                         | 61 |
| 2.2 Oligonucleotide primers and Enzymes .....                                    | 61 |
| 2.3 Maintenance and culture of mammalian cell, viral, and bacterial stocks.....  | 62 |
| 2.3.1 Maintenance of mammalian cell lines.....                                   | 62 |
| 2.3.2 hCMEC/D3 cells.....                                                        | 62 |
| 2.3.3 Storage of Viral Stocks .....                                              | 63 |
| 2.3.4 Storage of Bacterial Stocks .....                                          | 63 |
| 2.4 Molecular Techniques .....                                                   | 64 |
| 2.4.1 Nucleic Acid Extraction and Preparation Techniques.....                    | 64 |
| 2.4.1.1 Small Scale Plasmid Preparation from Bacterial Colonies .....            | 64 |
| 2.4.1.2 Large Scale Plasmid Preparation from Bacterial Colonies .....            | 64 |
| 2.4.1.3 Extraction of RNA from Virus Culture Supernatant .....                   | 64 |
| 2.4.1.4 Extraction of DNA from Agarose Gel Slices.....                           | 64 |
| 2.4.1.5 Extraction of DNA from PCR mix .....                                     | 64 |
| 2.4.2 Polymerase Chain Reaction (PCR).....                                       | 65 |
| 2.4.2.1 PCR Using Q5® DNA Polymerase.....                                        | 65 |
| 2.4.2.2 PCR for Bacterial Colony Screening.....                                  | 65 |
| 2.4.2.3 Reverse Transcriptase PCR.....                                           | 66 |
| 2.4.3 Storage of Nucleic Acids .....                                             | 67 |
| 2.4.4 Restriction Enzyme Digestion .....                                         | 67 |
| 2.4.5 Agarose Gel Electrophoresis.....                                           | 67 |
| 2.4.6 SDS-PAGE electrophoresis and Western Blot.....                             | 68 |
| 2.5 Sequencing.....                                                              | 68 |
| 2.5.1 Sanger Sequencing.....                                                     | 68 |

|        |                                                                                              |    |
|--------|----------------------------------------------------------------------------------------------|----|
| 2.5.2  | Next Generation Sequencing.....                                                              | 68 |
| 2.6    | Bacterial Transformation and Culture .....                                                   | 69 |
| 2.6.1  | Bacterial Transformation .....                                                               | 69 |
| 2.6.2  | Bacterial Culture on Agar Plates .....                                                       | 69 |
| 2.6.3  | Bacterial Culture in LB Broth .....                                                          | 69 |
| 2.7    | Nano-Glo® Luciferase Assay .....                                                             | 69 |
| 2.8    | NEBuilder® HiFi DNA Assembly .....                                                           | 70 |
| 2.9    | Q5® Site-Directed Mutagenesis.....                                                           | 71 |
| 2.10   | FuGENE transfection of DNA into cell culture .....                                           | 71 |
| 2.11   | Infection of cell culture with Fowlpox-T7 virus .....                                        | 72 |
| 2.12   | Rabies Virus Rescue and Culture .....                                                        | 72 |
| 2.12.1 | Rabies Virus Rescue from cDNA.....                                                           | 72 |
| 2.12.2 | Virus Titration .....                                                                        | 73 |
| 2.13   | Subculture of viral stocks to high titres .....                                              | 73 |
| 2.14   | Multi-Step Virus Growth Curves .....                                                         | 73 |
| 2.15   | Enzyme-linked Immunosorbent Assay .....                                                      | 74 |
| 2.16   | Cell culture fixation and RABV N immunocytochemistry .....                                   | 75 |
| 2.17   | Fluorescent Antibody Virus Neutralisation Test .....                                         | 75 |
| 2.18   | Fluorescent antibody test.....                                                               | 75 |
| 2.19   | Rabies tissue culture isolation test.....                                                    | 76 |
| 2.20   | MTS Cell Viability Assay .....                                                               | 76 |
| 2.21   | Assessing kinetics of cell-to-cell spread of rabies viruses .....                            | 76 |
| 2.22   | Using a hCMEC/D3 transwell assay to assess the ability of RABV-62scFv to cross the BBB ..... | 77 |
| 2.22.1 | Formation of the hCMEC/D3 BBB model.....                                                     | 77 |
| 2.22.2 | Assessing permeability of the hCMEC/D3 BBB model using Lucifer Yellow .....                  | 77 |
| 2.22.3 | Assessing the ability of RABV-62scFv to cross a hCMEC/D3 BBB model .....                     | 78 |
| 2.23   | Reverse genetics schematics and sequences of oligonucleotides .....                          | 78 |
| 2.23.1 | Generation of pTIT-NLuc construct to assess T7-polymerase expression.....                    | 79 |
| 2.23.2 | Generation of pNL1.1 sec62-NLuc and pNL1.1 62-NLuc.....                                      | 79 |
| 2.23.3 | Insertion of the 62-71-3 scFv ORF within RABV-cSN .....                                      | 79 |
| 2.23.4 | Replacement of DsRED of the 62-71-3 scFv ORF with mCHERRY .....                              | 79 |
| 2.23.5 | Mutation of RABV-cSN G protein at residue 226.....                                           | 79 |
| 2.23.6 | Alteration of the transcriptional start and stop units of the 62-71-3 scFv ORF .....         | 80 |
| 2.23.7 | Replacement of the RABV G gene in RABV-cSN with the IKOV G gene.....                         | 80 |
| 2.23.8 | Replacement of 62-71-3 with murine TNFα .....                                                | 80 |
| 2.23.9 | Removal of the 8xHis and E-Tag from the 62-17-3 scFv ORF within RABV-cSN .....               | 81 |
| 2.24   | Optimisation of virus rescue.....                                                            | 81 |
| 2.24.1 | Assessment of the human heavy chain SP.....                                                  | 81 |

|         |                                                                                                                                             |     |
|---------|---------------------------------------------------------------------------------------------------------------------------------------------|-----|
| 2.25    | <i>In Vivo</i> studies.....                                                                                                                 | 81  |
| 2.25.1  | Mice .....                                                                                                                                  | 81  |
| 2.25.2  | Viral Mouse Challenge and termination .....                                                                                                 | 82  |
| 2.25.3  | Serology .....                                                                                                                              | 82  |
| 2.25.4  | Formalin fixation of mouse carcasses.....                                                                                                   | 82  |
| 2.26    | Histopathology.....                                                                                                                         | 82  |
| 2.26.1  | Preparation of formalin-fixed mouse carcasses for histopathological analysis .....                                                          | 82  |
| 2.26.2  | Histopathological staining and imaging.....                                                                                                 | 82  |
| 2.27    | Analytic Software.....                                                                                                                      | 83  |
| 2.27.1  | Benchling .....                                                                                                                             | 83  |
| 2.27.2  | NEB Tm Calculator .....                                                                                                                     | 83  |
| 2.27.3  | NEBaseChanger® .....                                                                                                                        | 83  |
| 2.27.4  | NEBuilder® .....                                                                                                                            | 83  |
| 2.27.5  | OligoEvaluator™ .....                                                                                                                       | 83  |
| 2.27.6  | Promega Tm for Oligos Calculator .....                                                                                                      | 83  |
| 2.27.7  | SnapGene Viewer .....                                                                                                                       | 84  |
| 2.28    | Statistical Analysis.....                                                                                                                   | 84  |
| 2.29    | Addgene.....                                                                                                                                | 84  |
|         | Chapter 3 - Virus cloning and Rescue .....                                                                                                  | 85  |
| 3.1     | Introduction .....                                                                                                                          | 85  |
| 3.2     | Results.....                                                                                                                                | 86  |
| 3.2.1   | Generation of a Nanoluciferase expressing construct under the control of a T7-promoter .....                                                | 86  |
| 3.2.2   | Optimisation of T7-polymerase expression in Virus Rescue .....                                                                              | 86  |
| 3.2.3   | Generation of a nanoluciferase construct to assess the ability of a human heavy chain SP to promote secretion in BSR-T7/5 cell culture..... | 88  |
| 3.2.4   | Assessment of the human heavy chain SP in promoting secretion from mammalian cell culture .....                                             | 89  |
| 3.2.5   | Insertion, mutation, and replacement of elements within RABV-cSN for successful virus rescue .....                                          | 91  |
| 3.2.4.1 | Insertion of the 62-71-3 scFv cassette within the RABV genome.....                                                                          | 91  |
| 3.2.4.2 | Replacement of DsRED with mCHERRY for enhanced cell culture visualisation .....                                                             | 91  |
| 3.2.4.3 | Mutation of RABV-cSN G at site 226 to prevent self-neutralisation by 62-71-3 scFv.....                                                      | 92  |
| 3.2.4.4 | Alteration of the gene borders surrounding the 62-71-3 scFv cassette within RABV .....                                                      | 94  |
| 3.2.5   | Virus rescue of RABV-62-H-E-mk-Poly(A) .....                                                                                                | 95  |
| 3.2.6   | Investigating the failure of virus rescue .....                                                                                             | 97  |
| 3.2.6.1 | Is the cell death caused by RABV-62-H-E-mK-Poly(A) cell type specific? .....                                                                | 98  |
| 3.2.6.2 | Is residual binding of RABV G protein by vp62-71-3 the cause of cell death?.....                                                            | 99  |
| 3.2.6.3 | Replacement of the RABV G gene of RABV-cSN with the IKOV G gene.....                                                                        | 101 |

|                                                                                                                                 |     |
|---------------------------------------------------------------------------------------------------------------------------------|-----|
| 3.2.6.4 Is the cause of the apoptotic phenotype and cell death due to the 62-71-3 scFv protein?..                               | 102 |
| 3.2.6.5 Are the 8xHis-tag and E-tag responsible for the apoptotic phenotype associated with cell death? .....                   | 105 |
| 3.3 Discussion .....                                                                                                            | 107 |
| Chapter 4 - In Vitro Virus Characterisation.....                                                                                | 113 |
| 4.1 Introduction.....                                                                                                           | 113 |
| 4.2 Results.....                                                                                                                | 114 |
| 4.2.1 Assessing Cellular Toxicity of RABV-62scFv infection.....                                                                 | 114 |
| 4.2.2 Multi-Step Virus Growth Curves.....                                                                                       | 114 |
| 4.2.3 In vitro virus dissemination assay.....                                                                                   | 117 |
| 4.2.4 Production of 62-71-3 scFv over time .....                                                                                | 120 |
| 4.2.5 Identification of vp62-71-3 scFv using protein immunoblotting.....                                                        | 120 |
| 4.2.6 Investigating the ability of vp62-71-3 to bind RABV G protein using immunocytochemistry..                                 | 121 |
| 4.2.7 Assessing Neutralisation Activity of vp62-71-3 scFv using the Fluorescent Antibody Virus Neutralisation (FAVN) Test ..... | 123 |
| 4.2.8 Ability of RABV-62scFv to cross an <i>in vitro</i> Blood-Brain Barrier Model.....                                         | 125 |
| 4.3 Discussion .....                                                                                                            | 127 |
| Chapter 5 - In vivo assessment of RABV-62scFv .....                                                                             | 134 |
| 5.1 Introduction.....                                                                                                           | 134 |
| 5.2 Results.....                                                                                                                | 136 |
| 5.2.1 Toxicity assessment of RABV-62scFv .....                                                                                  | 136 |
| 5.2.1.1 Serological assessment of mice infected with RABV-62scFv .....                                                          | 138 |
| 5.2.1.2 Investigating the presence of RABV N protein and live virus in mouse brain .....                                        | 139 |
| 5.2.1.3 Histopathological assessment of CNS immune cell infiltration .....                                                      | 141 |
| 5.2.2 Assessment of RABV-62scFv as a post-exposure treatment .....                                                              | 142 |
| 5.2.2.1 Serological assessment of mice treated with RABV-62scFv.....                                                            | 146 |
| 5.2.2.2 Histopathological assessment of N protein presence in mice .....                                                        | 148 |
| 5.3 Discussion .....                                                                                                            | 152 |
| Chapter 6 - Discussion and Future Work .....                                                                                    | 159 |
| Chapter 7 - References.....                                                                                                     | 170 |
| Appendix .....                                                                                                                  | 190 |
| Appendix 1 - Standard Buffers and solutions .....                                                                               | 190 |
| Appendix 2 - Oligonucleotide Primers .....                                                                                      | 196 |
| Appendix 3 -Cloning Schematics .....                                                                                            | 198 |

## List of Figures

|                                                                                                                                                                                               |    |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| Figure 1:1: Schematic of the rabies virus and genome.....                                                                                                                                     | 28 |
| Figure 1:2: Schematic illustrating the division of the RABV G protein into the extracellular, transmembrane (TM), and cytoplasmic domain, alongside the location of six antigenic sites. .... | 32 |
| Figure 1:3: Showing the timeline of the virus lifecycle, and cellular receptors utilised, from muscle to peripheral nerve cells.....                                                          | 33 |
| Figure 1:4: Schematic of the nervous system, illustrating the layout of both sensory and motor neurone nerve pathways. ....                                                                   | 36 |
| Figure 1:5: A schematic of the BBB NVU and the adhesion molecules involved in the sealing of ECs together. ....                                                                               | 37 |
| Figure 1:6: A schematic of the pathway RABV takes during infection. ....                                                                                                                      | 39 |
| Figure 1:7: Illustration of signalling pathway of TLRs 3/7, RIG-I, and MDA-5 leading to the transcription of ISGs.....                                                                        | 43 |
| Figure 1:8: Interactions of rabies virus proteins with cells of the infected host to either evade or actively interfere with the activation of the immune system. ....                        | 47 |
| Figure 3:1: Schematic of the cloning process involved in the creation of the pTIT-NLuc vector. ....                                                                                           | 86 |
| Figure 3:2: Investigation of the optimal method of enabling T7 polymerase expression in mammalian cell culture.....                                                                           | 88 |
| Figure 3:3: Schematic illustrating the cloning involved in the production of pNL1.1 sec62-NLuc and pNL1.1 62-NLuc.....                                                                        | 89 |
| Figure 3:4: Investigating the effectiveness of the human heavy chain SP sequence in the secretion of 62-71-3 ScFv from BHK-21 cell culture. ....                                              | 90 |
| Figure 3:5: A schematic showing the 62-71-3 scFv cassette and its subsequent cloning strategy into the RABV-cSN backbone vector. ....                                                         | 91 |

|                                                                                                                                                                                                                                   |     |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| Figure 3:6: A schematic of the cloning strategy used to substitute the DsRED fluorophore for the mCHERRY fluorophore to enhance visualisation of infected cells in cell culture. ....                                             | 92  |
| Figure 3:7: A schematic illustrating the cloning strategy employed to induce a K226R mutation in the RABV glycoprotein gene of the pTIT-G vector, and its subsequent substitution into a linearised RABV-62-H-E-mCH vector.. .... | 93  |
| Figure 3:8: The cloning method employed for the re-insertion of the 62-71-3-mCHERRY cassette into the cSN (K226R-G) backbone. ....                                                                                                | 95  |
| Figure 3:9: Images showing viral rescue of RABV-62-H-E-mK-Poly(A) and RABV- EBLV-1-eGFP. ....                                                                                                                                     | 96  |
| Figure 3:10: 1% agarose gel showing a DNA band from RT-PCR of virus RNA extraction. ....                                                                                                                                          | 97  |
| Figure 3:11: BHK-21 cells infected with either CVS-11 (A/B), IKOV (C), or uninfected (D). ....                                                                                                                                    | 100 |
| Figure 3:12: Schematic illustrating the cloning method employed when substituting RABV-K226R-G for the IKOV G gene. ....                                                                                                          | 101 |
| Figure 3:13: A schematic of the cloning used to create two TNF $\alpha$ -expressing RABV constructs, RABV-TNF $\alpha$ -H-E-mK and RABV-TNF $\alpha$ (mem)-H-E-mK. ....                                                           | 103 |
| Figure 3:14: UV microscope images of RABV-TNF $\alpha$ -H-E-mK and RABV-TNF $\alpha$ (mem)-H-E-mK virus rescues. ....                                                                                                             | 105 |
| Figure 3:15: Schematic showing the cloning strategy involved in the creation of RABV-62scFv. ....                                                                                                                                 | 106 |
| Figure 3:16: UV microscope images of RABV-62scFv and RABV- EBLV-1-eGFP. ....                                                                                                                                                      | 107 |
| Figure 4:1: Assessing the effect of RABV-62scFv infection on mitochondrial respiration. ....                                                                                                                                      | 114 |
| Figure 4:2: Growth kinetics of RABV-cSN, RABV-mCH, RABV-mCH-K226R, and RABV-62scFv. ....                                                                                                                                          | 115 |
| Figure 4:3: Viral dissemination assay investigating the differences between the cellular dissemination of RABV-cSN, RABV-mCH, RABV-mCH-K226R, RABV-62scFv, CVS-11, and RABV-CVS-mCH over 120 h. ....                              | 118 |
| Figure 4:4: Representative images of foci generated in the cellular dissemination assay by either RABV-cSN, RABV-62scFv, or RABV-CVS-mCHERRY infection. ....                                                                      | 119 |

|                                                                                                                                                                               |     |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| Figure 4:5: vp62-71-3 scFv production over time after BHK-21 cell culture monolayer infection with RABV-62scFv. ....                                                          | 120 |
| Figure 4:6: Western Blot of supernatant from infected cell culture. Cells were infected with either RABV-mCH (A), RABV-mCH-K226R (B), RABV-62scFv (C), or RABV-cSN (D). ....  | 121 |
| Figure 4:7: CVS-11-infected BHK-21 cells fixed and stained for RABV N and G protein.....                                                                                      | 122 |
| Figure 4:8: RV437 infected BHK-21 cells fixed and stained for RABV N and G protein. ....                                                                                      | 123 |
| Figure 4:9: Kinetics of hCMEC/D3 BBB model formation over time. At each 24 h time point after cell seeding, an apparent permeability ( $P_{app}$ ) value was calculated. .... | 125 |
| Figure 4:10: Assessment of the presence of RABV-62scFv to cross a hCMEC/D3 BBB. ....                                                                                          | 126 |
| Figure 4:11: Images taken of a hCMEC/D3 blood-brain barrier monolayer to illustrate cellular infection with RABV-62scFv. ....                                                 | 127 |
| Figure 5:1: Survival curves of mice used to assess RABV-62scFv toxicity.....                                                                                                  | 137 |
| Figure 5:2: Serological assessment of mouse sera after challenge (ic or iv) with either RABV-62scFv or RABV-CVS-mCH, or media control. ....                                   | 139 |
| Figure 5:3: Histopathological assessment of mouse brains after ic challenge with RABV-CVS-mCH. ....                                                                           | 142 |
| Figure 5:4: A survival curve of mice used to investigate the applicability of RABV-62scFv as a post-exposure prophylaxis treatment.....                                       | 145 |
| Figure 5:5: Illustration of how RABV-62scFv treatment extended the symptomatic period of lethally challenged mice. ....                                                       | 146 |
| Figure 5:6: Representative images of histopathology and immunohistochemistry on brainstem of mice used to assess RABV-62scFv as a post-exposure prophylaxis treatment.. ....  | 151 |
| Figure 5:7: Representative images of Immunohistochemistry on the cerebrum of mice for RABV N protein.....                                                                     | 152 |
| Figure A1: 1% Agarose gel of PCR products used to create pTIT-NLuc.....                                                                                                       | 198 |

|                                                                                                                                                                       |     |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| Figure A2: 1% Agarose gels of PCR products used to assemble pNL1.1 sec62-NLuc and pNL1.1 62-NLuc. ....                                                                | 199 |
| Figure A3: Agarose gels of the PCR products involved in the creation of RABV-62-H-E-DsR. ....                                                                         | 200 |
| Figure A4: Agarose gels of the PCR products involved in the creation of RABV-62-H-E-mCH. ....                                                                         | 201 |
| Figure A5: An agarose gel of the PCR products used in the point mutation of amino acid 226 from K to R. ....                                                          | 202 |
| Figure A6: Agarose gels showing the PCR products involved in the re-insertion of the 62-71-3-mCHERRY cassette into RABV-cSN. ....                                     | 203 |
| Figure A7: Agarose gel showing the PCR products used in the creation of RABV-62-H-E-mCH-IKOV. ....                                                                    | 204 |
| Figure A8: A schematic of the cloning used to create two TNF $\alpha$ -expressing RABV constructs, RABV-TNF $\alpha$ -H-E-mK and RABV-TNF $\alpha$ (mem)-H-E-mK. .... | 205 |
| Figure A9: Agarose gel showing the PCR products involved in the creation of RABV-62scFv. ....                                                                         | 206 |

# List of Tables

|                                                                                                                                                       |     |
|-------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| Table 1:1: Current and historical types of rabies vaccines. ....                                                                                      | 50  |
| Table 1:2: A list of the various non-viral elements have been incorporated into the genome of LAVVs (Live Attenuated Virus Vaccines). ....            | 56  |
| Table 2:1: Cell culture media used in the maintenance of the different mammalian cell lines used in this thesis. ....                                 | 62  |
| Table 2:2: Components and final concentrations used in Q5® DNA polymerase reactions. ....                                                             | 65  |
| Table 2:3: Incubation times and temperatures used when performing PCR using the Q5® High-Fidelity 2X Master Mix. ....                                 | 65  |
| Table 2:4: Components and final concentrations used in GoTaq® Green PCR reactions used in screening for positive bacterial colonies. ....             | 66  |
| Table 2:5: Details of PCR timings and temperatures used in colony PCR using GoTaq® Green Master Mix. ....                                             | 66  |
| Table 2:6: Incubation times and temperatures used when performing reverse transcriptase PCR on viral RNA to create complementary DNA. ....            | 66  |
| Table 2:7: Components and final concentrations used in QIAGEN OneStep RT-PCR reactions. ....                                                          | 67  |
| Table 2:8: Components and volumes used in the NEB KLD reaction mix. ....                                                                              | 71  |
| Table 3:1: Assessment of the neutralising activity of 62-71-3 scFv DsRED (produced by <i>N. benthamiana</i> ) to both CVS-11 and IKOV. ....           | 101 |
| Table 4:1: Statistical differences between mean titres of RABV-cSN, RABV-mCH, RABV-mCH-K226R, and RABV-62scFv after plateauing in growth curves. .... | 116 |
| Table 4:2: Results of a FAVN test investigating the ability of vp62-71-3 scFv to neutralise both CVS-11 RABV and RV437. ....                          | 124 |
| Table 5:1: The ethical end point document used to score severity of RABV infection symptoms. ....                                                     | 137 |

|                                                                                                                                                                               |     |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| Table 5:2: Description of the dose, challenge route, survival, presence of N protein and live virus in mice used to assess RABV-62scFv toxicity. ....                         | 140 |
| Table 5:3: Description of ethical endpoints used to assess use of RABV-62scFv as a post-exposure treatment. ....                                                              | 143 |
| Table 5:4: Reciprocal serological titres and clinical scores of mice in each experimental group in the in vivo study assessing RABV-62scFv as a post-exposure treatment. .... | 148 |
| Table 5:5: Results of histopathological analysis of mouse brains and spinal cord for the presence of RABV N protein or pathological lesions (Ls).....                         | 150 |
| Table S1: Buffers and solutions used in this thesis. ....                                                                                                                     | 195 |
| Table S2: Sequences of oligonucleotide primers used in this project. ....                                                                                                     | 197 |

## Abbreviations

|                   |                                                                     |
|-------------------|---------------------------------------------------------------------|
| 3'                | Three prime                                                         |
| 5'                | Five prime                                                          |
| A (active)        | Active state                                                        |
| A (base)          | Adenine                                                             |
| aa                | Amino acid                                                          |
| ABLV              | Australian bat <i>Lyssavirus</i>                                    |
| Af-6              | Afadin-6                                                            |
| AJ                | Adherens junction                                                   |
| Ala               | Alanine                                                             |
| ANOVA             | Analysis of Variance                                                |
| APC               | Antigen presenting cell                                             |
| APHA              | Animal and Plant Health Agency                                      |
| AS- (e.g. AS-I)   | Antigenic site 1-5                                                  |
| Asn               | Asparagine                                                          |
| Asp               | Aspartic acid                                                       |
| ATCC              | American Type Culture Collection                                    |
| ATP               | Adenosine triphosphate                                              |
| ATP6V1A           | ATPase H <sup>+</sup> Transporting V1 Subunit A                     |
| ATV               | Antibiotic-trypsin-versene                                          |
| BBB               | Blood-brain barrier                                                 |
| BHK (e.g. BHK-21) | Baby hamster kidney                                                 |
| Bis-Tris          | Bis(2-hydroxyethyl)amine-Tris(hydroxymethyl)aminomethane            |
| BM                | Basement membrane                                                   |
| bp                | Base pair                                                           |
| BPL               | beta-Propiolactone                                                  |
| BSR-T7/5          | BHK-21 derivative cell line expressing T7 RNA polymerase            |
| BWA               | Burrows-Wheeler Alignment                                           |
| C (base)          | Cytosine                                                            |
| CA0               | Initial concentration of lucifer yellow added to apical compartment |
| CAP               | Cholinergic anti-inflammatory pathway                               |
| CARD              | Caspase-recruitment domains                                         |
| CCEEVs            | Cell culture and embryonated egg-based vaccines                     |

|                    |                                     |
|--------------------|-------------------------------------|
| CCL (e.g. CCL1)    | Chemokine ligand                    |
| CCR (e.g. CCR7)    | Chemokine receptor                  |
| CD (e.g. CD3)      | Cluster of differentiation          |
| cDNA               | Complementary DNA                   |
| CHO                | Chinese hamster ovary cell line     |
| cm                 | Centimetre                          |
| CMV                | Cytomegalovirus                     |
| CNS                | Central nervous system              |
| CO <sub>2</sub>    | Carbon dioxide                      |
| CSF                | Cerebrospinal fluid                 |
| cSN                | cDNA copy of SN RABV strain         |
| CSU                | Central sequencing unit at the APHA |
| CTD                | C-terminal domain                   |
| CVS (e.g. CVS-11)  | Challenge Virus Standard            |
| CxCL (e.g. CxCL10) | C-X-C motif chemokine ligand        |
| Da                 | Dalton                              |
| DAB                | 3,3' Diaminobenzidine               |
| DAMPs              | Damage associated molecular pattern |
| DC                 | Dendritic cell                      |
| DCBp               | DC-binding peptide                  |
| DIgA               | Dimeric IgA                         |
| DLC-1              | Deleted in liver cancer-1           |
| DMEM               | Dulbecco's Modified Eagle Medium    |
| DNA                | Deoxyribonucleic acid               |
| DRG                | Dorsal root ganglion                |
| DsR                | DsRED                               |
| dsRNA              | Double-stranded ribonucleic acid    |
| DUVV               | Duvenhage <i>Lyssavirus</i>         |
| E                  | Glutamic Acid                       |
| E. coli            | Escherichia coli                    |
| EBLV               | European bat Lyssavirus             |
| EC                 | Endothelial cell                    |
| ECL                | Enhanced chemiluminescence          |
| ECM                | Extracellular matrix                |

|                               |                                                    |
|-------------------------------|----------------------------------------------------|
| EDTA                          | Ethylenediaminetetraacetic acid                    |
| eGFP                          | Enhanced Green Fluorescent Protein                 |
| ELISA                         | Enzyme linked immunosorbent assay                  |
| EMEM                          | Eagle's minimum essential medium                   |
| ER                            | Endoplasmic reticulum                              |
| ER                            | Endoplasmic reticulum                              |
| ERA                           | Evelyn-Rokitnicki-Abelseth                         |
| ERIG                          | Equine rabies immunoglobulin                       |
| ESCRT                         | Endosomal sorting complexes required for transport |
| E-tag                         | Epitope tag                                        |
| FAT                           | Fluorescent antibody test                          |
| FAVN                          | Fluorescent antibody virus neutralisation test     |
| FBS                           | Foetal bovine serum                                |
| Fc                            | Fragment, crystallizable                           |
| FDC                           | Follicular dendritic cell                          |
| FFU                           | Focus-forming unit                                 |
| FITC                          | Fluorescein isothiocyanate                         |
| Fwd                           | Forward                                            |
| g                             | Gram                                               |
| G                             | Glycoprotein                                       |
| G (base)                      | Guanine                                            |
| GC                            | Germinal centre                                    |
| GC B cells                    | Germinal centre B cells                            |
| GFAP                          | Glial fibrillary acidic protein                    |
| GJ                            | Gap junction                                       |
| Gln                           | Glutamine                                          |
| Gly                           | Glycine                                            |
| GM-CSF                        | Granulocyte Macrophage Colony Stimulating Factor   |
| GMEM                          | Glasgow Minimum Essential Medium                   |
| h                             | Hour                                               |
| H <sub>2</sub> O              | Hydrogen dioxide (water)                           |
| H <sub>2</sub> O <sub>2</sub> | Hydrogen peroxide                                  |
| hCMEC/D3                      | Human cortical micro vessel endothelial cells/D3   |
| HDAC6                         | Histone deacetylase 6                              |

|                   |                                                           |
|-------------------|-----------------------------------------------------------|
| HDCV              | Human diploid cell vaccine                                |
| HEK-293T          | Human embryonic kidney 293T cells                         |
| HEP Flury         | High egg passage Flury strain of rabies virus             |
| HiFi              | High fidelity                                             |
| His               | Histidine                                                 |
| HMGB1             | High mobility group box protein 1                         |
| HPLC              | High-performance liquid chromatography                    |
| HRIG              | Human rabies immunoglobulin                               |
| HRP               | Horseradish peroxidase                                    |
| I                 | Inactive state                                            |
| Iba1              | Ionized calcium binding adaptor molecule 1                |
| ic                | intracranial                                              |
| ICV               | Intracerebroventricular                                   |
| IFIT (e.g. IFIT1) | Interferon-induced protein with tetratricopeptide repeats |
| IFN               | Interferon                                                |
| Ig (e.g. IgG)     | Immunoglobulin                                            |
| IGR               | Intergenic region                                         |
| IKOV              | Ikoma Lyssavirus                                          |
| IL (e.g. IL-5)    | Interleukin                                               |
| Ile               | Isoleucine                                                |
| im                | Intramuscular                                             |
| ip                | Intraperitoneal                                           |
| IRF (e.g. IRF7)   | Interferon Regulatory Factor                              |
| ISG (e.g. ISG15)  | Interferon-stimulated gene                                |
| ISRE              | Interferon-stimulated response elements                   |
| IU                | International units                                       |
| iv                | Intravenous                                               |
| IκB               | Inhibitor of nuclear factor kappa B                       |
| JAK1              | Janus Kinase 1                                            |
| JAM               | Junctional adhesion molecule                              |
| JHD               | Juvenile Huntington disease                               |
| K                 | Lysine                                                    |
| kb                | kilobase                                                  |
| kDa               | kilodalton                                                |

|                            |                                                                                                                         |
|----------------------------|-------------------------------------------------------------------------------------------------------------------------|
| KDKE                       | Lysine-Aspartic acid-Lysine-Glutamic acid                                                                               |
| kg                         | Kilogram                                                                                                                |
| KLD                        | Kinase ligase Dpn1                                                                                                      |
| ko                         | Knockout                                                                                                                |
| L                          | Large polymerase                                                                                                        |
| LAVVs                      | Live attenuated virus vaccines                                                                                          |
| LB                         | Luria Bertani                                                                                                           |
| LBV                        | Lagos bat virus                                                                                                         |
| LC8                        | Dynein light chain                                                                                                      |
| L-domain                   | Late-budding domain                                                                                                     |
| LDS                        | Lithium dodecyl sulphate                                                                                                |
| Le                         | Leader                                                                                                                  |
| LeRNA                      | Leader ribonucleic acid                                                                                                 |
| Leu                        | Leucine                                                                                                                 |
| LY                         | Lucifer yellow                                                                                                          |
| Lys                        | Lysine                                                                                                                  |
| M                          | Matrix                                                                                                                  |
| M (concentration)          | Molar                                                                                                                   |
| mAb                        | Monoclonal antibody                                                                                                     |
| MAC (e.g. MAC387)          | Macrophage Marker Antibody                                                                                              |
| MARVELD2                   | MARVEL [myelin and lymphocyte (MAL) and related proteins for vesicle trafficking and membrane link] domain containing 2 |
| MAVS                       | Mitochondrial antiviral signalling protein                                                                              |
| mCH                        | mCHERRY                                                                                                                 |
| MDA-5                      | Melanoma differentiation-associated protein 5                                                                           |
| MDC                        | Macrophage-derived chemokine                                                                                            |
| MEM                        | Minimum Essential Medium                                                                                                |
| mFAVN                      | Modified fluorescent antibody virus neutralisation                                                                      |
| Mfsd2a                     | Major Facilitator Superfamily Domain Containing 2A                                                                      |
| Mg                         | Milligram                                                                                                               |
| mGluR2                     | Metabotropic glutamate receptor 2                                                                                       |
| MHC                        | Major histocompatibility complex                                                                                        |
| min                        | Minute                                                                                                                  |
| MIP (e.g. MIP-1 $\alpha$ ) | Macrophage inflammatory protein                                                                                         |

|                |                                                                                              |
|----------------|----------------------------------------------------------------------------------------------|
| mL             | Millilitre                                                                                   |
| mM             | Millimolar                                                                                   |
| MN             | Motor neurone                                                                                |
| MOI            | Multiplicity of infection                                                                    |
| MOPS           | 3-(N-morpholino) propane sulfonic acid                                                       |
| MRC            | Medical research council 5                                                                   |
| mRNA           | Messenger ribonucleic acid                                                                   |
| MTase          | Methyltransferase                                                                            |
| MTS            | 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-<br>2H-tetrazolium |
| Mx1            | MX Dynamin Like GTPase 1                                                                     |
| N              | Nucleoprotein                                                                                |
| N. benthamiana | Nicotiana benthamiana                                                                        |
| N2a            | Neuronal-2a                                                                                  |
| nAChR          | Nicotinic acetylcholine receptor                                                             |
| NC             | Nucleocapsid                                                                                 |
| NCAM           | Neural cell adhesion molecule                                                                |
| NEB            | New England biolabs                                                                          |
| Nedd4          | Neuronal precursor cell-expressed developmentally downregulated 4                            |
| NES            | Nuclear export signal                                                                        |
| NFκB           | Nuclear factor kappa B                                                                       |
| ng             | Nanogram                                                                                     |
| Ni             | Nickle                                                                                       |
| NK             | Natural killer                                                                               |
| NLRP3          | NOD-, LRR- and pyrin domain-containing protein 3                                             |
| NLS            | Nuclear localisation signal                                                                  |
| NLuc           | Nanoluciferase                                                                               |
| nM             | Nanomolar                                                                                    |
| nm             | Nanometer                                                                                    |
| NMJ            | Neuromuscular junction                                                                       |
| No             | Monomeric nucleoprotein                                                                      |
| nt             | Nucleotide                                                                                   |
| NTD            | N (amine)-terminal domain                                                                    |
| N-terminal     | Amine-terminal                                                                               |

|                         |                                                                                                                                                                          |
|-------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| NVE                     | Non-viral element                                                                                                                                                        |
| NVU                     | Neurovascular unit                                                                                                                                                       |
| °C                      | Degrees Celsius                                                                                                                                                          |
| OD450                   | Optical density at 450 nm                                                                                                                                                |
| ORF                     | Open reading frame                                                                                                                                                       |
| OX40-L                  | Ligand for the OX40 receptor (Tumour necrosis factor receptor superfamily, member 4)                                                                                     |
| P                       | Phosphoprotein                                                                                                                                                           |
| P1-5                    | Phosphoprotein isoform variant 1-5                                                                                                                                       |
| p75NTR                  | p75 neurotrophin receptor                                                                                                                                                |
| PAMPs                   | Pathogen associated molecular pattern                                                                                                                                    |
| Papp                    | Permeability coefficient                                                                                                                                                 |
| Par- 3                  | Proteinase activated receptor 3                                                                                                                                          |
| PBS                     | Phosphate-buffered saline                                                                                                                                                |
| PCECV                   | Purified chick embryo cell vaccine                                                                                                                                       |
| PCR                     | Polymerase chain reaction                                                                                                                                                |
| pDC                     | Plasmacytoid dendritic cell                                                                                                                                              |
| PDEV                    | Purified duck embryo vaccine                                                                                                                                             |
| PDZ                     | Domain named after the first three proteins to share this domain: Post synaptic density protein, Drosophila disc large tumour suppressor, and Zonula occludens-1 protein |
| pen-strep               | Penicillin-streptomycin                                                                                                                                                  |
| PEP                     | Post-exposure prophylaxis                                                                                                                                                |
| pH                      | Power of hydrogen                                                                                                                                                        |
| pH                      | Power of hydrogen                                                                                                                                                        |
| Phe                     | Phenylalanine                                                                                                                                                            |
| PHKCV                   | Primary hamster kidney cell vaccine                                                                                                                                      |
| PIT                     | Post-infection treatment                                                                                                                                                 |
| PML                     | promyelocytic leukaemia                                                                                                                                                  |
| pmol                    | Picomole                                                                                                                                                                 |
| PNS                     | Peripheral nervous system                                                                                                                                                |
| PrEP                    | Pre-exposure prophylaxis                                                                                                                                                 |
| PRNTasae capping enzyme | Polyribonucleotidyltransferase capping enzyme                                                                                                                            |

|                 |                                                                           |
|-----------------|---------------------------------------------------------------------------|
| PRR             | Pattern recognition receptors                                             |
| PSD             | Postsynaptic density                                                      |
| PVRV            | Purified Vero cell rabies vaccine                                         |
| R               | Arginine                                                                  |
| RABV            | Rabies virus                                                              |
| Rag-2           | Recombination activating gene 2                                           |
| RdRp            | Ribonucleic acid-dependent RNA polymerase                                 |
| RelA            | v-rel reticuloendotheliosis viral oncogene homolog A                      |
| Rev             | Reverse                                                                   |
| RFP             | Red fluorescent protein                                                   |
| rh EGF          | Recombinant human epidermal growth factor                                 |
| RIG             | Rabies immunoglobulin                                                     |
| RIG-I           | Retinoic acid-inducible gene I                                            |
| RLRs            | Retinoic acid-inducible gene I (RIG-I)-like receptors                     |
| RLU             | Relative luciferase unit                                                  |
| RNA             | Ribonucleic acid                                                          |
| RNP             | Ribonucleoprotein                                                         |
| ROS             | Reactive oxygen species                                                   |
| rpm             | Revolutions per minute                                                    |
| RT              | Room temperature                                                          |
| RTCIT           | Rabies tissue culture isolation test                                      |
| RT-PCR          | Reverse transcription polymerase chain reaction                           |
| RVGp            | Rabies virus glycoprotein peptide                                         |
| SAD             | Street Alabama Dufferin                                                   |
| SAG (e.g. SAG1) | SAD Avirulent Gif                                                         |
| SARS-COV-2      | Severe acute respiratory syndrome coronavirus 2                           |
| ScFv            | Single-chain fragment variable                                            |
| SDS-PAGE        | Sodium dodecyl sulphate (SDS) - polyacrylamide gel electrophoresis (PAGE) |
| sec             | Second                                                                    |
| Ser             | Serine                                                                    |
| SHBRV           | Silver-haired bat rabies virus                                            |
| SN              | Sensory neurone                                                           |
| SOC             | Super optimal broth with catabolite repression                            |
| SP              | Signal peptide                                                            |

|                    |                                                                                                                          |
|--------------------|--------------------------------------------------------------------------------------------------------------------------|
| STAT (e.g. STAT-1) | Signal transducer and activator of transcription                                                                         |
| T                  | Thymine                                                                                                                  |
| T705               | Favipiravir, 6-fluoro-3-hydroxy-2-pyrazinecarboxamide                                                                    |
| TAE                | Tris-Acetate EDTA (Ethylenediaminetetraacetic acid)                                                                      |
| TBK1               | TANK [TRAF (Tumour Necrosis Factor Receptor–Associated Factor) Family Member Associated NFKB Activator] Binding Kinase 1 |
| TBS                | Tris [Tris(hydroxymethyl)aminomethane]-buffered saline                                                                   |
| TCID50             | 50 % Tissue culture infective dose                                                                                       |
| TCR                | T cell receptor                                                                                                          |
| Tfh                | T follicular helper cell                                                                                                 |
| TGFβ               | Transforming growth factor-β                                                                                             |
| Th (e.g. Th1)      | T helper                                                                                                                 |
| TJ                 | Tight junction                                                                                                           |
| TLR (e.g. TLR3)    | Toll-like receptor                                                                                                       |
| TM                 | Transmembrane                                                                                                            |
| TM                 | Transmembrane                                                                                                            |
| Tm                 | Melting temperature                                                                                                      |
| TMB                | 3,3',5,5'-Tetramethylbenzidine                                                                                           |
| TNFα               | Tumour necrosis factor alpha                                                                                             |
| Tr                 | Terminal repeat                                                                                                          |
| Treg               | Regulatory T cell                                                                                                        |
| Tris               | Tris(hydroxymethyl)aminomethane                                                                                          |
| Tyr                | Tyrosine                                                                                                                 |
| U                  | Unit                                                                                                                     |
| UV                 | Ultraviolet                                                                                                              |
| VB                 | Volume in the basolateral compartment                                                                                    |
| VH                 | Variable heavy                                                                                                           |
| VL                 | Variable light                                                                                                           |
| VNA                | Virus neutralising antibodies                                                                                            |
| vp62-71-3          | Virus-produced 62-71-3                                                                                                   |
| vRNA               | Viral ribonucleic acid                                                                                                   |
| vRNP               | Viral nucleoprotein                                                                                                      |
| VSV                | Vesicular stomatitis virus                                                                                               |
| WHO                | World Health Organisation                                                                                                |

|                            |                                                                                 |
|----------------------------|---------------------------------------------------------------------------------|
| x g (e.g. 100 x g)         | Centrifugation force                                                            |
| ZO (e.g. ZO-1)             | Zonula occludens                                                                |
| $\Delta\text{CB}/\Delta t$ | Change in lucifer yellow concentration over time in the basolateral compartment |
| $\Delta\text{XF}$          | Lacking fucose and xylose glycosylation                                         |
| $\mu\text{g}$              | Microgram                                                                       |
| $\mu\text{M}$              | Micromolar                                                                      |
| $\mu\text{L}$              | Microliter                                                                      |
| $\mu\text{m}$              | Micrometer                                                                      |
| $\mu\text{mole}$           | Micromole                                                                       |

# Chapter 1 - INTRODUCTION

## 1.1 RABIES VIRUS

Rabies, caused by all members of the *Lyssavirus* genus, is a significant zoonosis affecting both human and animal populations globally with an estimated 59,000 human deaths annually <sup>1-3</sup>. The *Lyssavirus* genus belongs to the *Rhabdoviridae* family of viruses, and as such, Lyssaviruses contain a negative-sense single-stranded RNA genome encoding five viral genes: N (nucleoprotein), P (phosphoprotein), M (matrix), G (glycoprotein), and L (large polymerase) <sup>4,5</sup>. Rabies virus (RABV) is the prototypic virus of the *Lyssavirus* genus with well-defined epidemiology in some areas for both volant and non-volant mammal species. However, the epidemiology of the remaining Lyssaviruses, 16 distinct viral species that have been most often reported from bat species, remain largely undefined with often only a few isolates being available for each species <sup>6</sup>.

Rabies is an economically important zoonosis where its economic burden results from the treatment of illness, human deaths, and mass vaccination campaigns in multiple animal species <sup>7</sup>. These vaccination campaigns have been effective in eliminating wildlife reservoirs and dog-mediated rabies in multiple developed countries in Western Europe and the Americas <sup>8,9</sup>. However, in developing countries where rabies vaccination campaigns are not a priority, domestic dogs (*Canis lupus familiaris*) remain the primary reservoir <sup>7</sup>. Furthermore, because of the different cultural mentalities around responsible dog ownership and control, human infections occur [and, therefore, post-exposure prophylaxis (PEP) expenditure is required] and continents such as Asia and Africa bear a disproportionate economic burden compared to developed countries, the majority of which comes from premature deaths, direct costs of PEP, and lost income while seeking PEP <sup>3,7,10</sup>.

### 1.1.1 The structure and shape of the RABV

Rabies virions consist of proteins, lipids, carbohydrates, and RNA. The wild type viral genome contains approximately 12 kilobases (kb) of RNA which is coiled tightly around N, P, and L proteins to form the ribonucleoprotein (RNP) complex. RNP is packaged alongside matrix (M) proteins within a host-derived lipid envelope containing viral G proteins <sup>11</sup>. Interestingly, while 98% of virions contain negative sense viral RNA (vRNA), approximately 2% of particles have been reported to contain positive-sense vRNA <sup>12,13</sup>. Together, these assemble to form a bullet-shaped virion (under the effect of the M protein) of approximately 60 × 180 nm (Figure 1:1) <sup>14</sup>.

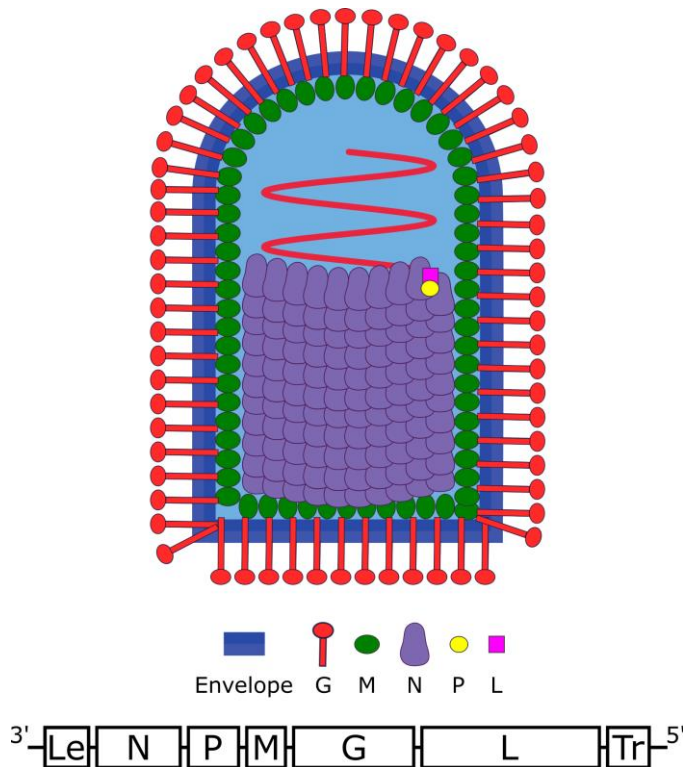


Figure 1:1: Schematic of the rabies virus and genome. The rabies virion shows glycoproteins (G) embedded into the host cell-derived envelope. These Gs are in association with the matrix (M) proteins, which further interact with the internal RNP complex. RNP consists of RNA - encapsidating nucleoprotein (N), phosphoprotein (P), and Large polymerase (L) protein. The rabies genome schematic shows the order these genes are encoded, in addition to the 3' leader (Le) and 5' terminal repeat (Tr).

## 1.2 PROTEINS OF RABV

### 1.2.1 N protein

The rabies N protein is the most abundantly produced protein during RABV replication and is one of the most conserved protein between Lyssaviruses<sup>15</sup>. This high degree of conservation can be attributed to the vital role N protein plays in both viral replication and transcription. At every stage of viral replication, RNA is complexed with N protein in a ring-like assembly of 9 nucleotides associated with a single N protein molecule<sup>16</sup>. The RABV N protein is comprised of approximately 450 amino acids (aa) and can be separated into an N- (NTD) and C-terminal domain (CTD). Here, the N-terminus interacts with RABV P, while the cleft between the NTD and CTD represent the RNA binding cavity (aa 289-352)<sup>16-19</sup>. Within this RNA binding cavity, five basic residues coordinate the N-RNA interaction<sup>11</sup>. RABV N protein is also phosphorylated at serine (Ser) 389 by a cellular kinase during the RABV replication cycle<sup>20</sup>. When unphosphorylated, N binds strongly to genomic RNA. To avoid unwanted host RNA binding, N

protein forms a complex with P protein shortly after production. The subsequent encapsidation of viral RNA promotes a conformational change in the N-P complex, allowing Ser389 phosphorylation which weakens N-RNA interactions to allow L protein access to begin transcription and replication <sup>17,20-23</sup>.

### 1.2.2 P protein

The RABV P gene is situated directly downstream of the N gene and may be translated from any one of five in-frame start codons of its mRNA. This results in a full-length P protein (P1) and four less-abundant isoforms (P2/3/4/5) <sup>24</sup>. The rabies P protein (approximately 300 aa) has a crucial role in viral replication, mediating interactions between N and L proteins. Isoforms P2-5 lack the ability for L protein interaction and are predominantly involved in immune evasion which will be discussed later <sup>25</sup>. Due to the presence of multiple CTD and NTD nuclear localisation and nuclear export signals, P protein isoforms exist in a balance of nuclear export and import. Specifically, P1/2 isoforms contain an NTD and CTD nuclear export signal (NES), in addition to a CTD nuclear localisation signal (NLS) which results in their strong localisation to cell cytoplasm. The NTD truncation involved in the translation of P3 results in the abolishment of the NTD NES but reveals an additional NTD NLS. This, in concert with the CTD NES and NLS results in strong nuclear localisation. Lastly, P4/5 lack this new NTD NLS and only the CTD NLS and NES signals, resulting in a diffuse but predominantly nuclear localisation <sup>26,27</sup>.

The RABV P protein, like the N, is also phosphorylated and exists in two forms depending on the location of phosphorylation. While the function of phosphorylation is yet to be fully revealed, it may be involved in the switch between RABV transcription and genome replication <sup>28</sup>. As discussed previously, P binds N shortly after translation to modulate and direct RNA binding but also to prevent monomeric N protein (N<sup>o</sup>) self-assembly. RABV P protein has two sites available for N protein binding, the first situated on the CTD, and the second on the N-terminal half <sup>18,23</sup>. These, respectively, are involved in the binding of the N-RNA complex and binding of N<sup>o</sup> <sup>22</sup>. During the formation of RNP, P protein is also required to interact with L protein to facilitate formation of a functioning RNA polymerase complex. This interaction occurs using the N-terminal region of the P protein. Furthermore, similar to conclusions drawn from studies assessing vesicular stomatitis virus (VSV) P protein, RABV P protein also enhances both the initiation and processivity of L protein <sup>29</sup>.

Lastly, an LC8 (dynein light chain) binding domain is present within the RABV P protein. LC8 is a subunit of the cytoplasmic dynein motor complex <sup>30</sup>, and early studies envisioned mechanisms where the ability of P protein to bind LC8 would facilitate retrograde axonal trafficking along neuronal cells. However, mutational studies revealed that removal of the LC8 binding domain

did not abolish retrograde tracking. However, these studies did reveal that LC8 binding domain eradication reduced early transcription and replication <sup>31</sup>. RABV P protein has also been described to associate with other host proteins, including the L9 component of the 60S ribosome (able to modulate RABV replication) <sup>32</sup>, mitochondrial Complex I (involved in reactive oxygen species (ROS) which may induce neuronal injury) <sup>33</sup>, and focal adhesion kinase (aiding viral replication) <sup>34,35</sup>.

### 1.2.3 M protein

The rabies M gene, which encodes the M protein, is located at position three within the RABV genome and contributes to viral replication in multiple aspects <sup>36,37</sup>. The M protein both binds and condenses viral nucleocapsid (NC) and recruits these NCs for assembly into virions at the cell surface. This was demonstrated in studies investigating the effect of removing the M gene from the RABV genome, which reported severely impacted viral budding and caused the generation of long rod-shaped virions <sup>14</sup>. The ability of M protein to direct budding of virions relies upon the presence of late domain (L-domain) motifs. These L-domain motifs interact with members of the endosomal sorting complexes required for transport (ESCRT) pathway to facilitate recruitment of NCs to cell surface. In studies where late-domains have been disrupted, virions were unable to fully separate from the host cell <sup>38</sup>. While the M protein may be the primary determinant for viral budding, the efficient budding of RABVs relies on the collaborative effort of both the M and G proteins <sup>14</sup>. Here, the cytoplasmic tail of the RABV G protein is able to interact with the RNP-coating M protein, an observation supported by the lack of colocalisation seen between G protein and RNPs lacking the M protein <sup>14</sup>. While the precise G-binding domain on the M protein has not been identified, a highly acidic domain spanning aa 115-151 has been shown to be essential <sup>39</sup>.

In addition to the critical role of the M protein in viral budding, it also has vital roles in the regulation of viral transcription and replication. While exact mechanisms are unknown, the ability of the M protein to condense RNP inhibits vRNA transcription and prompts a shift from vRNA transcription to genome replication <sup>40</sup>. M protein is also able to upregulate histone deacetylase 6 (HDAC6), allowing depolymerisation of the microtubule skeleton (microtubules are almost undetectable in RABV infected cells) <sup>41</sup>. This depolymerisation results in the formation of tubulin monomers which have been hypothesised to bind M protein and promote release from RNP to allow transcription and replication <sup>41</sup>. Lastly, as stated previously, the M protein is tightly associated with RNP within mature virions: upon cellular infection it must disassociate from RNP to allow vRNA transcription and replication. This cytoplasmic disassociation from RNP was previously believed to be a passive process, however, protein interactome studies have demonstrated that interaction of RABV M protein with V-type

proton ATPase catalytic subunit A (ATP6V1A) promotes the disassociation of the M protein from RNP <sup>42</sup>.

#### 1.2.4 G protein

Rabies has a single type I transmembrane envelope G, a 65 kilodalton (kDa) protein comprised of four domains: signal peptide (SP), ectodomain, transmembrane (TM), and a cytoplasmic domain. The G protein is present as a trimer on the RABV surface and has roles in viral assembly, mediating cell attachment, and cell entry through the binding of surface cell receptors and pH-induced membrane fusion <sup>43-46</sup>.

After translation, the N-terminal SP domain first facilitates the embedding of G protein into the endoplasmic reticulum (ER)-Golgi Apparatus membrane, where it remains fixed by its TM domain, before it is transported to the plasma membrane and SP cleaved <sup>47</sup>. While the process has not been thoroughly studied in RABV, equivalent studies using VSV suggest trimerisation is one of the final steps prior to exit from the ER as the majority of G proteins present on the Golgi and cell surface are within trimers <sup>48-52</sup>. Due to distinct morphological and antigenic differences, RABV G protein trimerisation is vital and relies upon a sequence of hydrophobic bases within the TM domain. However, this TM domain does not necessarily need to be of RABV origin <sup>53,54</sup>.

Upon appropriate folding and trimerisation of the G protein, as discussed previously the cytoplasmic domain of G protein is able to interact with RNP-M complexes to promote viral assembly and eventual release <sup>14</sup>. Interestingly, while G contributes to assembly and release, these processes can occur without it (albeit with a 30-fold reduction in efficiency) <sup>14,55</sup>.

##### 1.2.4.1 Cell membrane fusion activities of the RABV G.

The RABV G protein occupies three different conformational states: the native, active, and fusion-inactive state. While the native and active states are antigenically similar, the RABV G protein in the fusion-inactive state is distinct and more extended than the native state <sup>56</sup>. At neutral pH, RABV G protein is in the native state. Immediately upon acidification, this switches to the active state and prolonged incubation in an acidic environment induces the fusion-inactive state <sup>57</sup>. Unlike other viruses, transition between fusion states is reversible upon incubation at different pH. This is beneficial, as during cellular synthesis RABV G protein trimers occupy the fusion-inactive state (due to the low pH of the Golgi and subsequent transport vesicles) to avoid any unnecessary membrane fusion <sup>57,58</sup>.

During cellular infection, the RABV G protein occupies different states at different stages. Cell entry is mediated by the binding of RABV G, in the native state, to cell receptors including nicotinic acetylcholine receptor (nAChR), neural cell adhesion molecule (NCAM), p75

neurotrophin receptor (p75NTR), metabotropic glutamate receptor 2 (mGluR2), and Integrin  $\beta 1$ <sup>43,44,59</sup>. These receptors are discussed in greater detail in Section 1.2.4.2. Upon receptor binding, RABV is endocytosed in clathrin-coated pits in both neuronal and non-neuronal cells. Due to the size of RABV, these partially coated pits also require actin polymerisation to fully endocytose from the plasma membrane<sup>60</sup>. After endocytosis, RABV-containing endosomes gradually acidify<sup>60,61</sup> where RABV G protein undergoes a morphological change to the active state. Once here, active-state G protein can interact with endosomal membranes (13-19 Gs are required) and promote membrane fusion for viral nucleoprotein (vRNP) release into host cytoplasm<sup>42,56</sup>.

Due to the major role of the RABV G protein in infection, and its presence on the surface of RABV virions, G protein is a major immunological target. Antibody mapping studies have identified approximately six antigenic sites (AS) within the RABV G protein. These include; AS-I, located at aa 226-231 with both discontinuous and continuous epitopes; AS-II, comprising two discontinuous sites (aa34-42 and aa192-200); AS-III, a continuous epitope, located at aa330-338; minor site a, at aa342-343; AS-IV, located at aa251; and AS-V at aa261-264<sup>62,63</sup>. A schematic is shown in Figure 1:2.

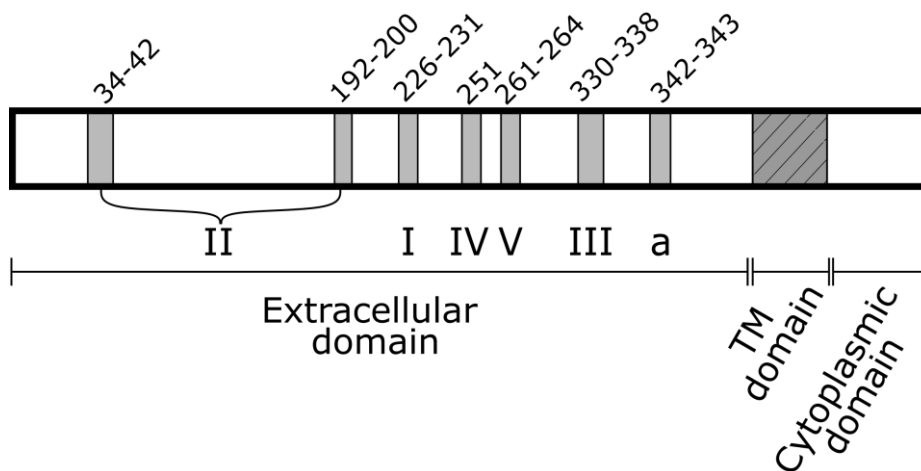


Figure 1:2: Schematic illustrating the division of the RABV G protein into the extracellular, transmembrane (TM), and cytoplasmic domain, alongside the location of six antigenic sites.

#### 1.2.4.2 Receptors for viral infection

Five receptors have been proposed for the cellular entry of RABV, including nAChR, NCAM, p75NTR, mGluR2, and Integrin  $\beta 1$ <sup>2,43,44,59</sup> (Figure 1:3). nAChRs are receptors for the nicotinic acetylcholine neurotransmitter and are present on the post-synaptic muscle membrane of neuromuscular junctions (NMJ). While nAChRs are also present throughout the central (CNS) and peripheral (PNS) nervous system, the subunit that RABV G protein binds to,  $\alpha 1$ , is only present on muscle cells and consequently nAChR forms one of the entry receptors for muscle

cells<sup>43</sup>. Furthermore, nAChR may also concentrate RABV virions within the synapse for efficient presentation to neuronal attachment receptor NCAM (otherwise known as CD56) and P75NTR, both expressed at nerve synapses/NMJs<sup>2,43</sup>. Interestingly, p75NTR has been shown to be non-essential for RABV infection<sup>64</sup>. mGluR2 and Integrin  $\beta 1$  are the two most recently discovered receptors for RABV. Similar to nAChR, mGluR2 is a neurotransmitter receptor, which is highly expressed within the CNS. RABV binding to mGluR2 has been demonstrated to induce internalisation into early and late endosomes<sup>59</sup>. Meanwhile, Integrin  $\beta 1$  allows signal interaction between the extracellular matrix and intracellular components and thus is involved in a multitude of processes including tumorigenesis, cell adhesion and proliferation<sup>44,65,66</sup>.

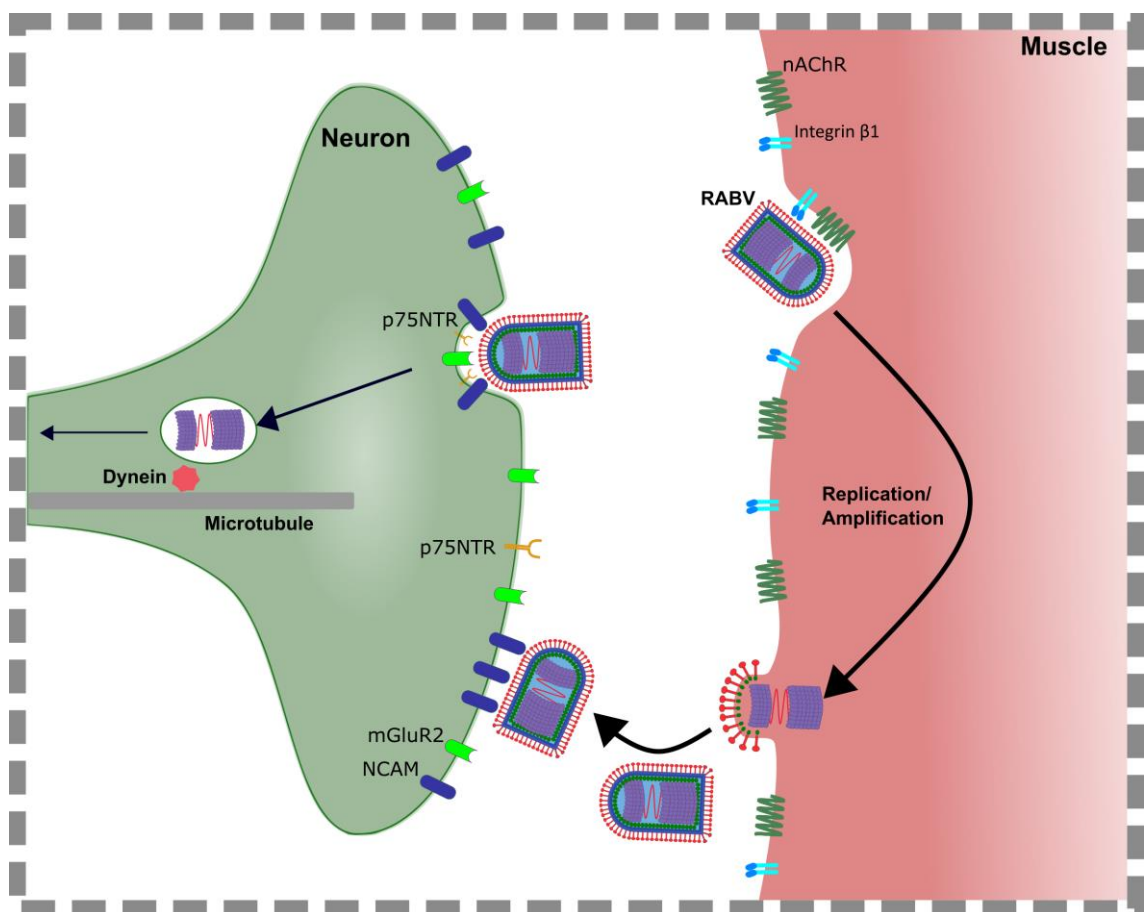


Figure 1:3: Showing the timeline of the virus lifecycle, and cellular receptors utilised, from muscle to peripheral nerve cells.

### 1.2.5 L Protein

The rabies L protein is the 5' terminal gene within the RABV genome and contains a number of enzymatic properties including being an RNA dependent RNA polymerase (RdRp). After the necessary association with both the N and P proteins of the viral RNP, the L protein performs all the enzymatic activities needed for both transcription and replication. The enzymatic activities L protein is able to perform include transcription, replication, capping, and

polyadenylation<sup>67</sup>. By structural and functional comparison to the VSV L protein, the RABV L protein has been proposed as being organised in the following sequence from N to C domains: N-terminal domain (NTD), RNA-dependent RNA polymerase (RdRp), DLC-1 binding bridge, Polyribonucleotidyltransferase (PRNTase) capping enzyme, connector domain, methyltransferase (MTase) and a CTD. Furthermore, the RdRp domain has been subdivided into finger, palm, and thumb subdomains and multiple structural motifs<sup>5,68-70</sup>. Together, these domains interact during RABV infection to effectively allow the transcription of RABV genes before switching to genome replication.

### 1.3 LIFE CYCLE OF RABV

When a host is bitten by a RABV infected animal, RABV can be transmitted deep into the affected tissue. This may involve initial infection of the musculature or immediate entry into the PNS or CNS, depending on the location of the bite.<sup>71</sup> Upon muscle cell infection, a period of low-level replication is assumed to occur in sub-optimal cellular environments. This period of low-level replication may contribute to inordinately long prodromal periods following exposure, although a variety of factors are thought to be involved including wound severity, wound location (proximity to the CNS), dose of inoculated virus, and virus strain<sup>72</sup>. Virus activity in the host during pre-clinical incubation periods remains unclear<sup>73,74</sup>. In cases where a high virus load infects muscle tissues during a bite, replication within muscle cells is bypassed and peripheral nerves may become infected<sup>75</sup>. Following receptor binding and acidification of cellular endosomes<sup>59,61,76</sup>, conformational changes in the RABV G protein promotes membrane fusion and RABV RNP is released into the cytoplasm. Here, cytosolic viral transcription and replication occurs (in late infection these processes occur in cytoplasmic inclusion bodies called Negri bodies) and new virions are assembled and released extracellularly<sup>77</sup>.

vRNA transcription begins when the RABV L protein, linked to both the P and N proteins present as the RNP complex, binds the 3' end of the RABV genome and transcribes a positive-sense leader (Le) RNA (LeRNA) and five messenger RNAs (mRNA) (N, P, M, G, and L), which are subsequently translated by the host cell machinery. The two 3' and 5' untranslated regions in the RABV genome, Le and the terminal repeat (Tr) respectively, facilitate the binding of the viral polymerase to either end of the RABV genome. In addition to signalling encapsidation by RABV N proteins, LeRNA also aids in the generation of both monocistronic (transcription) and full-length antigenome vRNAs (replication), while Tr allows the generation of genomic vRNA from the full-length antigenome vRNA<sup>78</sup>.

Between each open reading frame (ORF) of the viral genome is an intergenic region (IGR) comprising a polyadenylation signal (<sup>A</sup>/<sub>T</sub>G(A)<sub>7</sub>), an un-transcribed region (C(N)<sub>1-23</sub>), and the

transcriptional start sequence for the following gene (AACAC<sub>T</sub><sup>C</sup>/T<sub>T</sub><sup>C</sup>NCU)<sup>79</sup>. As the polymerase transcribes across these IGRs, a population of transcriptase complexes will disassociate, leading to a transcriptional gradient with 3' proximal genes being transcribed with transcriptional abundance reducing across the genome in a sequential manner resulting in genes towards the 5' end of the genome, such as the L protein, being least abundant within infected cells<sup>80</sup>.

As the concentration of intracellular viral proteins increase during transcription, the viral transcriptional L-P complex switches from transcription to replication. During the switch to replication, the viral transcriptase complex binds the Le region and ignores the distinct intergenic signals to transcribe a full-length positive-sense antigenome strand RNA, which is simultaneously encapsidated by N proteins. From this nascent positive antigenome RNA molecule, the viral N, P, and L proteins initiate replication at the antigenome Tr promoter to generate nascent full-length negative strand genome sense RNA which is also N protein encapsidated during production to prevent degradation by host cell factors. The switch between transcription and replication is poorly defined although a role for the M protein has been suggested. At low levels of M protein, transcription is favoured, while at high levels replication is favoured<sup>40</sup>.

The budding and release of nascent RABV virions is entirely dependent on the RABV M protein, with the viral glycoprotein playing a supportive role. Once the nascent RNP has been formed, M is able to bind this complex and facilitate condensation<sup>14</sup>. Simultaneously, G protein localises to the plasma membrane following translation through the rough ER and Golgi apparatus, forming sites for viral budding. RNP-M complexes then localise to these areas, with increased concentrations of M protein promoting RNP condensation. As membrane curvature begins, additional M protein is recruited into the condensing virion. Cellular complexes such as ubiquitin ligases and Neuronal precursor cell-expressed developmentally downregulated 4 (Nedd4) promote the recruitment of ESCRT complexes to the plasma membrane to facilitate budding and viral release and to completely separate from the cellular membrane<sup>81</sup>.

## 1.4 STRUCTURE OF THE BRAIN AND NERVOUS SYSTEM

The nervous system can be divided into the PNS and CNS. The PNS contains both sensory and motor neurons (MN) (See Figure 1:4). MNs may be defined as either upper or lower, and lower motor neurons may be either somatic or autonomic. While autonomic MNs innervate non-voluntary muscles such as the heart, somatic MNs are involved in the voluntary control of skeletal muscles<sup>82,83</sup>. All signals for skeletal muscle movement must originate from the brain and these signals are initially carried by the upper MNs, whose cell bodies are located in the

primary motor cortex of the brain. Upper MNs extend from the brain and through the spinal cord until it synapses with lower MNs. The signal is then carried by lower MNs where it effects MN-innervated muscle <sup>84</sup>. RABV preferentially infects somatic MNs <sup>85</sup> using various cell surface receptors discussed in Section 1.2.4.2.

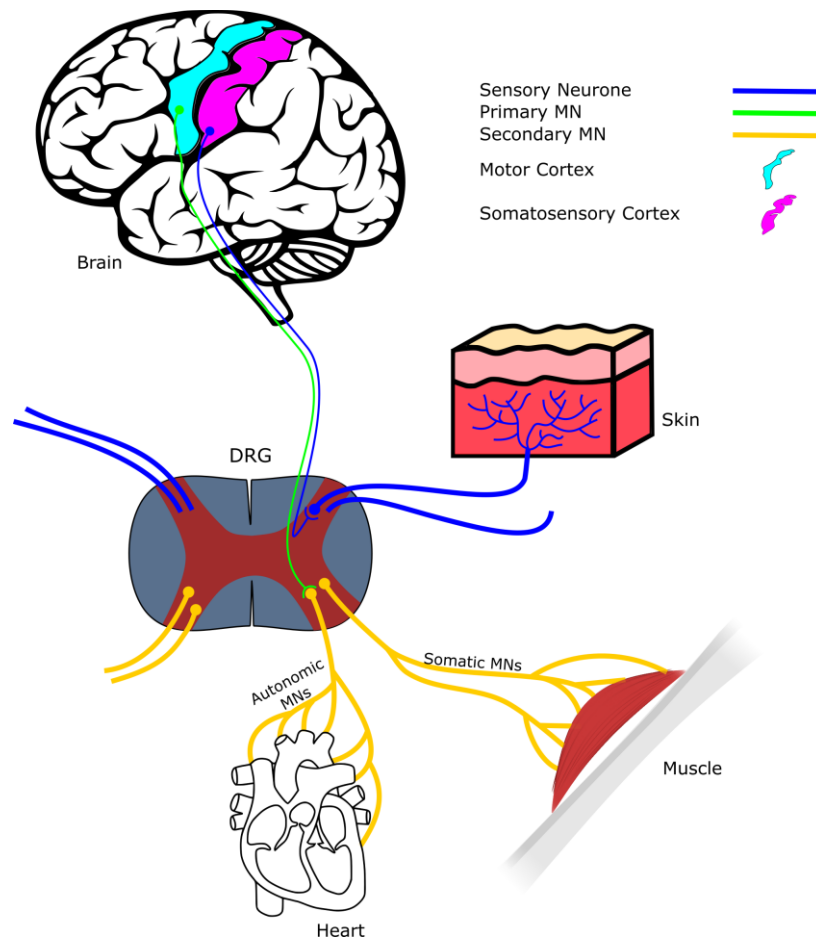


Figure 1:4: Schematic of the nervous system, illustrating the layout of both sensory and motor neurone nerve pathways. DRG= Dorsal root ganglion. MN= Motor neurone.

In contrast to MNs, sensory neurones (SN) function in the transmission of peripheral sensory (e.g. touch/feel) input to the brain. The cell bodies of these (pseudounipolar) SNs, alongside a larger population of glia, are located in the dorsal root ganglion (DRG) of the spinal cord. The DRG is a bilateral structure of the spinal cord, found at each vertebra and is surrounded by a thin layer of cerebrospinal fluid (CSF) <sup>86</sup>. SNs extend both to the periphery and the dorsal column nuclei of the brainstem, allowing the transmission of peripheral signals to the brain <sup>87</sup>.

#### 1.4.1 The blood-brain barrier and neurovascular unit

The blood-brain barrier (BBB) is a physical cellular barrier encompassing the endothelial cells of the CNS vasculature, acting to separate the brain/CNS from macromolecules [ $>400$  daltons (Da) <sup>88</sup>] in the blood. Made up primarily of endothelial cells (EC), the BBB also comprises

astrocytes and pericytes. Together, these form the neurovascular unit (NVU) (Figure 1:5). Due to the molecular size exclusion limit of the BBB ( $>400$  Da <sup>88</sup>), the BBB is able to block the migration of harmful biologics, such as RABV, from the blood to the brain. However, the BBB is also a major obstacle to the immune system in the treatment of RABV infection <sup>89</sup>. Specifically, peripherally generated anti-RABV antibodies are unable to access RABV within the CNS <sup>89</sup>.

As shown in Figure 1:5, ECs within the BBB are tightly sealed together by an array of inter-cell tight junctions (TJ), adherens (AJ), and gap junctions (GJ) <sup>90,91</sup>. The proteins of TJs, AJ, and GJs all interact with intracellular scaffolding proteins such as Zonula occludens-1/2 (ZO-1/2), afadin-6 (Af-6), and proteinase activated receptor-3 (Par-3) by their PDZ domains <sup>92</sup>. Together, these scaffolding proteins aid in the formation, assembly, and association with the cellular cytoskeleton of junctional complexes. While in some diseases BBB breakdown is associated with a deteriorating prognosis, in RABV infection BBB permeabilisation is associated with survival. Infection with attenuated RABV strains has repeatedly been shown to result in decreased TJ protein expression <sup>93</sup>. Interestingly, in contrast to other viruses, the permeabilisation of the BBB and downregulation of TJ components is not due to a direct action of a RABV protein but from the enhanced immune activation and release of interleukins (e.g. interleukin (IL) -5/6/12/17), interferons (IFN) (e.g. IFN $\gamma$ ), and chemokine ligands (e.g. CCL1-5/10/11) at the BBB surface <sup>93-95</sup>.

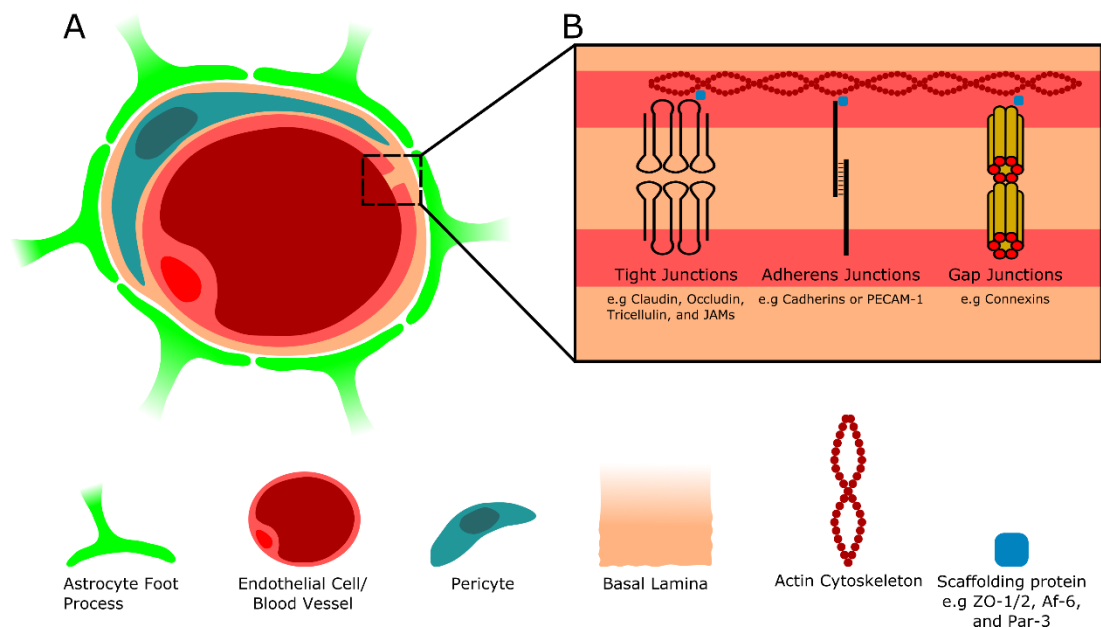


Figure 1:5: A schematic of the BBB NVU and the adhesion molecules involved in the sealing of ECs together. As shown in A, blood vessels in the CNS are encapsulated in both pericytes and astrocyte foot processes. These cells are situated in the basal lamina, and the ECs tightly sealed by tight junctions, adherens junctions, and gap junctions (B). TJs are comprised of transmembrane proteins such as claudin, occludin, tricellulin, and junctional adhesion

molecules (JAM). Where tight junctions tightly seal the spaces between ECs, adherens junctions act in the initiation of EC-EC adhesion. Cadherins are the dominant transmembrane proteins involved in adherens junction formation, interacting with other cadherin molecules on neighbouring ECs. Lastly, and in contrast to tight junctions and adherens junctions, gap junctions allow the migration of small molecules and ions between cells, allowing intercellular communication.

The basement membrane (BM) is a proteinaceous anchor for the cellular components of the NVU. The BM is 50-100 nm thick and is comprised of four extracellular matrix proteins: collagen, laminin, nidogen, and perlecan, produced by ECs, pericytes and astrocytes. In the NVU, the BM may be defined as either the endothelial or parenchymal BM, separated by pericytes. However, in the absence of pericytes these BM layers are indistinguishable <sup>96</sup>.

As explored by Brown et al <sup>97</sup>, pericytes are an important cellular component of the NVU and play multiple essential roles within the BBB and vasculature of the brain/CNS. Embedded within the BM, pericytes are able to actively contract and relax to control blood flow through ECs due to the presence of alpha-smooth muscle actin. Pericytes also direct the EC cell cycle and pericyte-deficient mice show reduced vasculature within the brain, suggesting a role in the development of blood vessels and capillaries within the CNS. Alongside producing components of the BM, pericytes are also able to maintain the integrity of the BBB by increasing TJ expression and reducing EC transcytosis through major facilitator super family domain containing 2a (Mfsd2a) EC induction <sup>98</sup>.

The last component of the NVU are astrocytes. Astrocyte cellular processes extend to and coat neuronal processes and blood vessels, giving them a vital role in signalling to the CNS <sup>99</sup>.

Astrocyte foot processes encapsulate BBB blood vessels with direct interactions with the BM <sup>90</sup>. Astrocytes have been demonstrated to modulate CNS water, ion, aa, and neurotransmitter homeostasis, and interactions with pericytes allow blood flow regulation. Astrocytes are also able to maintain BBB integrity by multiple mechanisms through the use of sonic hedgehog and transforming growth factor- $\beta$  (TGF $\beta$ ) <sup>90,99</sup>. *In vitro* studies have demonstrated that RABV is, in principle, able to infect astrocytes <sup>100,101</sup>. However, *in vivo* studies have shown that the ability of attenuated RABV strains to infect astrocytes is reliant upon the route of infection where [in contrast to intracranial (ic) infection] intramuscular (im) infection is unlikely to result in astrocyte infection due to rapid immune activation and clearance. Conversely, due to the ability of pathogenic RABV strains to avoid activation of the immune system, astrocyte infection is seen (late infection if administered peripherally) regardless of route of inoculation

<sup>102</sup>.

Together, the features of the NVU discussed above result in an effective barrier, separating molecules in circulation from the CNS and brain parenchyma<sup>103</sup>. This separation also plays a major role in infection by forming a physical barrier to protect the brain<sup>104</sup>. While the permanent opening of the BBB is associated with many pathologies, a transient increase in permeability has been shown to be a major factor in CNS clearance of RABV as this facilitates infiltration of both antibodies and immune cells<sup>94,105-108</sup>. However, pathogenic strains of RABV have been shown to employ various mechanisms to counteract the opening of the BBB and the subsequent infiltration of immune cells (discussed in more detail in Section 1.5)<sup>107,109</sup>.

#### 1.4.2 Viral dissemination within the host and the onset of clinical disease

Once RABV has infected MNs of the PNS, virions spread transynaptically in a retrograde fashion towards the CNS and brain (Figure 1:6A/B)<sup>76,110</sup>. While less common, RABV has also been observed to infect SNs<sup>85</sup>. Once infected, RABV may migrate along these cells to the spinal column and DRG. From here, RABV is then free to replicate further or spread to the brain or opposing contralateral DRG (Figure 1:6C)<sup>111</sup>. Importantly, during the late stages of infection, RABV is not limited to retrograde cell spread. Active G-dependent anterograde spread, albeit by undefined mechanisms, has been observed in DRG neurons and in mice<sup>112,113</sup>. It is this anterograde spread which allows the spread of RABV from the DRG to peripheral organs<sup>111</sup> or along facial nerves to the mandibular glands, where the salivary glands may then be infected and live virus excreted into the oral cavity (Figure 1:6C/D)<sup>114</sup>.

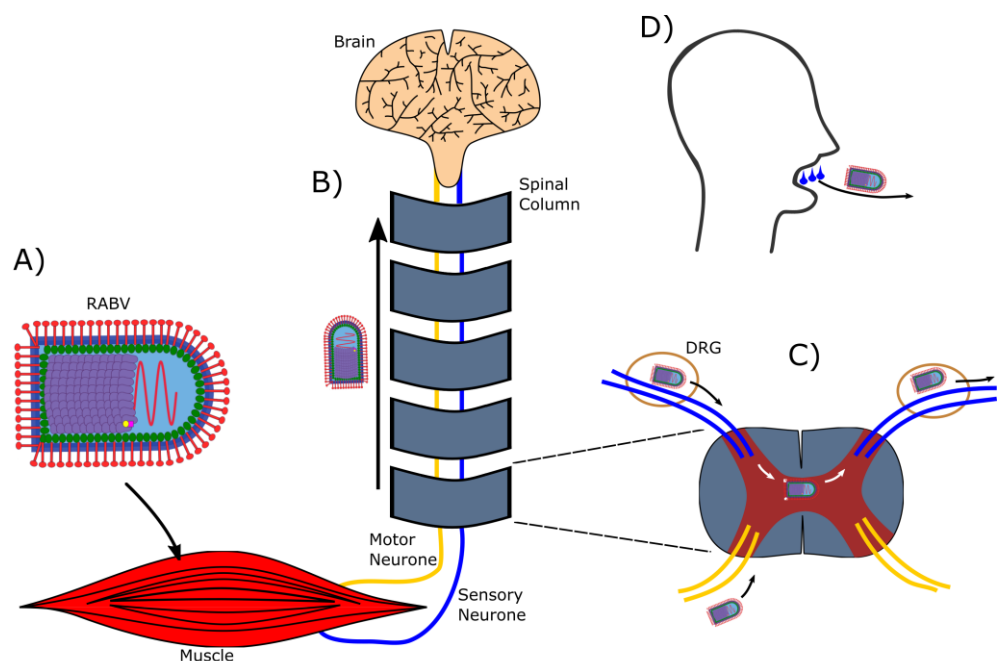


Figure 1:6: A schematic of the pathway RABV takes during infection. A, first RABV is inoculated deep into the muscle cells of the infected host organism. B, after a variable incubation period, RABV infects predominantly the motor neurones, but also the sensory neurones, where it is

trafficked towards the CNS and brain. C, within the spinal cord, RABV is also able to infect sensory neurones of the dorsal root ganglion (DRG) and, by anterograde spread, infect the contralateral DRG where it may spread along sensory neurones to further infect other organs. D, once RABV has reached the brain, further anterograde spread will allow RABV to spread along facial nerves to the salivary glands where RABV will be excreted.

#### 1.4.3 Symptoms of RABV infection

Patients infected with RABV undergo a number of clinical stages of infection. After an initial asymptomatic incubation stage, patients enter a prodromal period. Here, symptoms can be as non-specific as pruritus, paraesthesia, fever, fatigue, cough, nausea, vomiting, headache, or anxiety <sup>115</sup>. Patients then progress to show classical symptoms of rabies infection in the acute neurologic period, where patients present in one of two forms: either furious (encephalitic, which occurs in two-thirds of patients) or paralytic. Furious rabies is characterised by alternating periods of agitation/confusion and lucidity. Further symptoms include the production of tears, excessive sweating, and hydrophobia (which causes both fear and inspiratory muscle spasm). These muscle spasms are initially triggered by attempts to drink water but, as clinical symptoms progress, may also be triggered by aerophobia or the mention of water. Typically, patients exhibiting furious rabies die within a week.

Paralytic rabies is characterised by initial paraesthesia and weakness in the infection site. As clinical disease progresses, headaches, fever, bladder involvement, and general limb-weakness occur, before paralysis spreads to all limbs and respiratory muscles. Patients who present with paralytic rabies typically survive approximately 11 days <sup>116,117</sup>.

### 1.5 IMMUNE RESPONSE TO RABV INFECTION

The immune response to RABV infection is a complex interplay between the innate and adaptive arms of the immune system. In studies with an attenuated RABV strain, following infection of muscle a local innate immune response is activated, including the recruitment, activation, and maturation of dendritic cells (DC), macrophages, and natural killer (NK) cells. Here, antigen presenting cells (APC) such as DCs and macrophages are effective in phagocytosing RABVs, and their pattern recognition receptor (PRR)-mediated recognition of pathogen or damage associated molecular patterns (PAMP and DAMP, respectively) is essential in the induction of a type-I IFN response. NK cells are also able to mediate the recognition and killing of RABV-infected cells as well as the production of multiple IFN <sup>118-120</sup>. Once activated, DCs are able to migrate to lymph nodes to activate the maturation of T cells, which are skewed to elicit a humoral anti-RABV response. Directed by the action of chemokines, activated T and B cells then migrate to the site of infection <sup>121,122</sup> where they are

able to clear infection. However, pathogenic RABV strains exhibit multiple methods of either actively suppressing immune activation or effectively evading detection <sup>123</sup>. This results in reduced DC or macrophage activation at the initial site of infection and pathogenic RABV is able to replicate unimpeded <sup>118</sup>, spreading to the CNS, brain, and peripheral organs. Clearance of RABV from the CNS, an immunologically unique site due to the presence of multiple modulatory immune mechanisms and the presence of the BBB <sup>124</sup>, has been shown to be reliant on strong activation of the humoral adaptive immune response <sup>105,125,126</sup>.

### 1.5.1 Innate Immune response to RABV infection

Among the first cells to respond to the presence of RABV in the periphery are APCs such as macrophages and DCs. Historically, it was believed that muscle and neuronal cells were the only targets for RABV infection following the bite from an infected animal. However, further research has demonstrated that attenuated RABV strains are able to readily infect, replicate within, and activate macrophages and DCs. In contrast, pathogenic RABV strains have been reported to only able to bind and internalise within some macrophages and DC cell lines *in lieu* of replication <sup>118,127-129</sup>. The mechanism involved in how RABV is able to enter APCs such as DCs and macrophages in the absence of replication requires further study. This phenomenon of poor DC replication has also been observed in other negative stranded RNA viruses such as influenza <sup>130</sup>. DCs are 'professional APCs' and are able to phagocytose foreign bodies such as viruses quickly and present their T cell epitopes in the context of major histocompatibility complex (MHC) class I or II <sup>131</sup>. Upon PAMP recognition by PRR, DCs become activated and undergo changes in their ability to: capture new antigens, express levels of MHC molecules, and express co-stimulatory molecules <sup>131</sup>. Furthermore, an increase in chemokine receptor molecules (such as CCR2/5) will allow DCs to exit peripheral tissues and migrate to lymph nodes where they may activate T cells and bias the resulting immune response by a combination of signals <sup>132</sup>. These signals include the presentation of the processed peptide by the T cell receptor (TCR), the binding of costimulatory CD28 receptor to DC-expressed CD80/86 <sup>133</sup>, and the profile of cytokines released alongside the DC-T cell interaction. Together, these signals bias naïve T cells towards particular T cell phenotypes including Th1, Th2, Th9, Th17, Th22, Th25, Th40, Tfh and Treg cells <sup>131,134</sup>. While the importance of the adaptive immune response in rabies infection will be discussed later, it is important to note that a bias towards a Th1 immune response is essential for survival after rabies infection <sup>135</sup>. This is, in part, due to the characteristic expression of IFN $\gamma$  by Th1 CD4<sup>+</sup> cells which aids in the maturation of naïve DC cells and generation of inflammatory (M1) macrophages <sup>136-138</sup>.

#### 1.5.1.1 Pattern recognition receptors of the innate immune system

Based upon the detection of PAMPs or DAMPs by PRRs, the innate immune response is activated immediately after infection and elicits the release of IFNs, cytokines, and chemokines into the affected tissues<sup>118</sup> (Figure 1:7). Important PRRs with respect to RABV include toll-like receptors (TLRs) and retinoic-acid inducible gene (RIG)-like receptors (RLRs). TLRs are a family of 13 PRRs, while RLRs encompass RIG protein 1 (RIG-I) and melanoma differentiation-associated gene-5 (MDA-5). Depending on the PRR, these may either be situated within the cytoplasm (RLRs) or within endosomes (TLR3, 7-9, 13). Furthermore, these PRRs recognise different targets. Of importance to the innate immune response against RABV infection is the detection of viral RNA. Cytoplasmic RLRs recognise double stranded RNA, while endosomal PRRs such as TLR3 and 7 specialise in the recognition of double or single stranded RNA, respectively<sup>139-142</sup>. TLR7 is predominantly expressed within plasmacytoid DCs (pDC) and B cells, acts as one of the primary sensors for RNA viruses, and is vital in the formation of germinal centres (GC) essential for the generation of a humoral immune response<sup>143</sup>. TLR7 has also been implicated in causing excessive inflammation where TLR7<sup>-/-</sup> knockout (ko) mice show greater resistance to RABV after intracranial challenge<sup>144</sup>. The ligand required for TLR3 (double stranded RNA) activated is produced during RABV replication<sup>145</sup>. Upon activation, TLRs dimerise and trigger signalling cascades which result in the activation of transcription factors such as IFN-regulatory factor 7 (IRF7) or nuclear factor- $\kappa$ B (NF $\kappa$ B) which, upon translocation to the nucleus, bind IFN-stimulated response elements (ISREs) to promote the transcription of IFN-stimulated genes (ISG) involved in type I/III IFN responses<sup>146-148</sup>. Upon ligand binding, the N-terminal caspase-recruitment domains (CARD) of RLRs RIG-I and Mda-5 recruit mitochondrial antiviral-signalling protein (MAVS) which results, again, in the downstream activation of IRF3, 7 and NF $\kappa$ B for the stimulation ISREs for type I/III IFN responses<sup>147-149</sup>. Furthermore, infection of DCs and macrophages with attenuated RABV strains have been shown to activate NLR family pyrin domain containing 3 (NLRP3) inflammasome signalling, triggering the release of inflammatory IL-1 $\beta$  cytokine production and pyroptosis<sup>150,151</sup>.

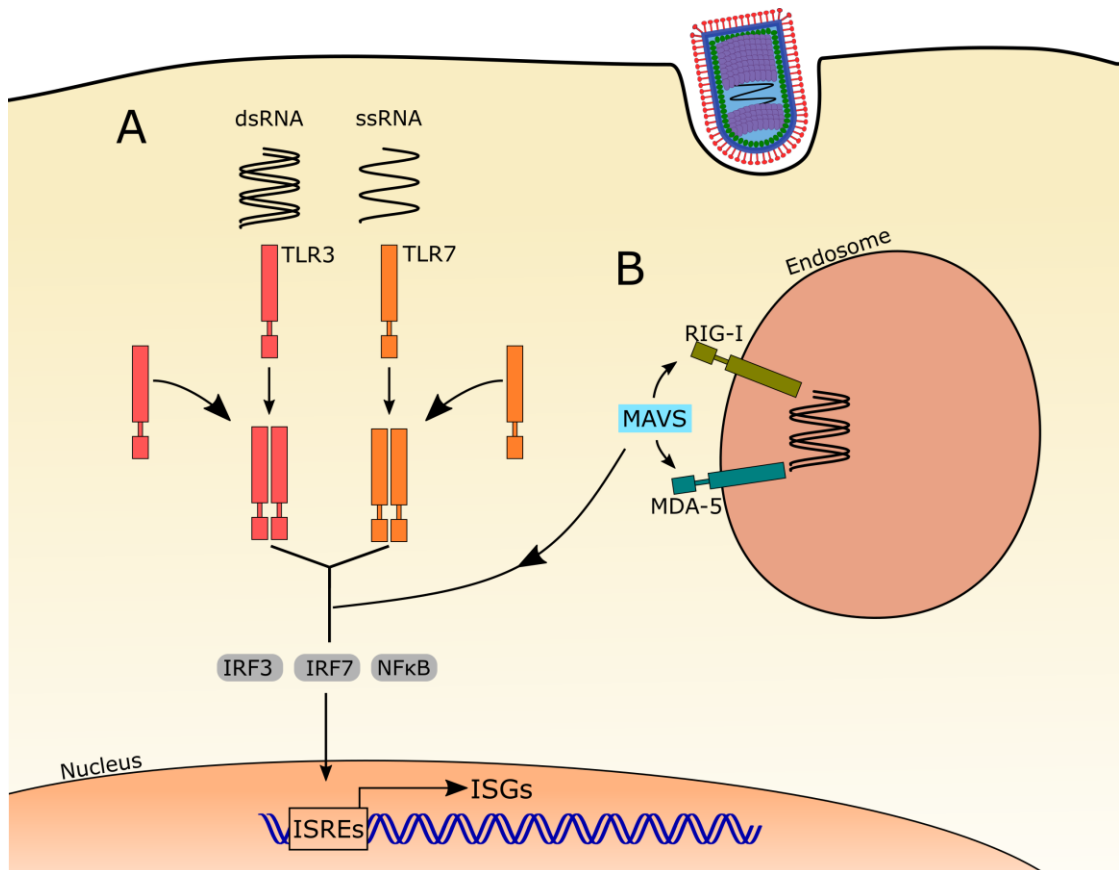


Figure 1:7: Illustration of signalling pathway of TLRs 3/7, RIG-I, and MDA-5 leading to the transcription of ISGs. A, receptors such as TLR3 and 7 are present in the cytoplasm of cells and recognise dsRNA and ssRNA respectively. After binding, these TLRs dimerise and lead to the activation of proteins including IRF3 and 7, and NFκB which enter the nucleus of the host cell to bind ISREs for the transcription of ISGs. Similarly, B shows endosome-localised RLRs RIG-I and MDA-5 which both recognise dsRNA. After activation, these receptors recruit MAVS which, similar to TLR3 and 7, lead to the activation of proteins including IRF3, IRF7, and NFκB which promote ISG transcription.

#### 1.5.1.2 Innate immunity in the brain

Upon exposure, RABV is able to infect both the cells present at the site of infection (usually muscular) but also within the PNS and CNS. Unsurprisingly, while an inflammatory immune response is generally detrimental within the CNS it is not an immune privileged site and possesses mechanisms needed for combating infection <sup>152,153</sup>.

During the resting state of the CNS *in vivo*, TLR expression is scarcely detectable (with the exception of TLR3 in rodent brains) but during infection is highly upregulated. Non-neuronal cells in the CNS, known as glia, are the dominant sources of TLR within the CNS. Microglia (CNS resident APCs) constitutively express TLRs 1-9, with the expression of TLRs 1-4 most dominant <sup>154</sup>, and astrocytes (shown to express TLRs 1-5 and 9, albeit at a lower level than microglia

<sup>154,155</sup>) are the major initiators and mediators of inflammation <sup>156</sup>. Within primary cultures of both human and mouse astrocytes, and in resting mouse brain, TLR3 is present in highest amount and undergoes the greatest fold-increase upon RABV infection <sup>154,156,157</sup>. This high baseline expression level of TLR3 may represent an 'early warning system' to common RNA virus infections, where increased expression levels allow an early response. Overall, the presence of TLRs on microglia, astrocytes, and neurons within the CNS allow the initiation of both a strong type-I and III IFN response to virus challenge, both of which have been shown to be essential for protection of *in vivo* models <sup>128,158,159</sup>.

Microglia are the main APC within the CNS but unlike DCs are unable to be the sole activator of naïve T cells. Furthermore, microglia have not been observed to migrate outside of the brain to draining lymph nodes <sup>160</sup>. For the initial activation of T cells after CNS infection, antigens are first transported to areas such as the choroid plexus either by passively draining across the leaky choroid plexus capillaries or being actively transported by microglia <sup>160-162</sup>. Once activated and antigen-bearing microglia are present within the choroid plexus, microglia may pass their presented peptide to DCs and promote DC activation. In cases where viral proteins drain to areas such as the choroid plexus without microglia, DCs may phagocytose these antigens and become activated. Activated DCs may then migrate to lymph nodes where they present antigens to promote T cell activation and maturation <sup>160</sup>. These T cells are then recruited to the CNS by the production chemokines (such as CXCL10) and integrins, predominantly by astrocytes and microglia <sup>121</sup>.

### 1.5.2 The adaptive immune response to RABV infection

DCs, upon activation, migrate to lymphoid tissues. Here, as discussed previously, an MHC-II – TCR interaction between DCs and naïve CD4<sup>+</sup> T cells (in addition to costimulatory signals) enables T cell maturation. Depending on the cytokine/co-stimulatory signal context of MHC-II – TCR interaction, DCs are able to bias the direction of T cell maturation into various T cell subtypes including Th1, Th2, Th9, Th17, Th22, Th25, Th40, Tfh and Treg cells <sup>134</sup>. Within the context of RABV infection, a Th1-based immune response (primarily directed against intracellular pathogens and the activation of cell-mediated immunity) within the CNS is strongly linked with survival <sup>135,163</sup>. In contrast to Th2 responses (antibody-mediated immunity and coordination of B cells) which are biased by the presence of IL-4 during DC-T activation, the Th1 response requires the presence of IL-12 <sup>164</sup>. A Th1 immune response is characterised by the production of IFN $\gamma$  by Th1 CD4<sup>+</sup> T cells, promoting the class switching of B cells to the production of IgG2a <sup>135</sup>. However, studies assessing Th1 responses in IFN- $\alpha/\beta$  receptor ko mice reveal that survival after RABV challenge is not dependent on antibody subclass, rather, CD4<sup>+</sup> cells with a Th1 bias are able to provide B cell help within the CNS <sup>135</sup>.

Alongside the activation of T cells within lymph nodes, activated DCs also play a vital role in the generation of high-affinity B cell responses within germinal centres. Following antigen recognition by DCs in the periphery, activated (TLR7 dependent <sup>144</sup>) and antigen laden DCs, upon reaching the lymph node, both present their antigens to naïve B cells and pass antigen to long-lived follicular DCs (FDC) through a variety of mechanisms <sup>165,166</sup>. Here, activated B cells upregulate their CCR7 receptor and migrate to lymphoid T cell zones where they present antigen to CD4<sup>+</sup> cells which promotes division and either the formation of GCs or differentiation into extrafollicular plasma cells (the initial source of antibodies upon infection) <sup>167</sup>. The presence of these GCs within B cell regions of lymph nodes allows the identification of primary (absent of GCs) and secondary follicles (presence of GCs). In these secondary follicles, FDC and Tfh cells aid in the generation of high-affinity B cell responses <sup>133,168-170</sup>.

#### 1.5.2.1 Importance of humoral immunity in RABV clearance from the CNS.

The innate and adaptive arms of the immune system are both vital to mounting an effective immune response. The adaptive immune response can broadly be defined into either a predominantly CD8<sup>+</sup> cytotoxic T cell response, or an antibody-dependent B cell response. Depending on the foreign agent, the immune system preferentially skews the immune response towards one of these two arms. For example, survival and clearance of hepatitis B/C virus, and multiple respiratory infections such as respiratory syncytial virus, influenza virus, and coronavirus all depend heavily on CD8<sup>+</sup> T cell responses <sup>171-173</sup>.

An effective immune response to RABV infection requires both B- and T-cell involvement <sup>105,135</sup>. This finding is supported by studies utilising Rag-2 (Recombination activating gene 2) and JHD (Juvenile Huntington disease) ko mice. Rag-2 ko mice lack both B and T cells, while JHD ko mice lack B cells only. Here, Rag-2 mice quickly succumb to infection while JHD ko mice are able to control RABV infection for an extended period <sup>105,125</sup>. This necessity of a B-cell humoral response has been confirmed in other gene ko studies where mice unable to generate an antibody response showed 100% mortality after attenuated virus challenge <sup>126</sup>.

During the course of natural infection, anti-RABV antibodies [particularly of the immunoglobulin (Ig) G isotype] have frequently been found circulating in the blood supply and CSF of lethally infected animals and humans <sup>95,174-176</sup>. While the mechanism of the antibody-mediated protection against RABV infection has not been thoroughly studied, neutralising antibodies may lock G protein in its pre-fusion state, preventing membrane fusion and cellular infection <sup>177</sup>. However, many studies have demonstrated that only small amounts of RABV-specific antibody cross the BBB during pathogenic RABV infection due to BBB permeability only increasing before a peak in serum antibody titre <sup>105,106,178</sup>. This increase in BBB permeability is

mediated by the production of chemokine CXCL10 by glial and neuronal cells. This recruits CD4<sup>+</sup> T cells which may differentiate into either Th17 (which produce IL-17 to promote BBB permeabilisation) or IFN $\gamma$ -producing Th1 cells<sup>93-95</sup>.

However, while neutralising antibodies are vital in the clearance of RABV within the CNS, it is the production of these high-affinity anti-RABV antibodies (which occurs during the peak of BBB permeabilisation) within the CNS by infiltrating B cells which is the main contributor to viral clearance<sup>94,105,126,179</sup>. This has been shown in studies investigating the immune response to attenuated RABV strain challenge virus standard-F3 (CVS-F3) strain, where CVS-F3 was shown to be cleared from the CNS not by infiltrating antibodies but by antibodies produced from infiltrating B cells. Lethal RABV [silver haired bat rabies virus (SHBRV)] has also been shown to be cleared from infected mice upon the promotion of CNS inflammation to allow infiltration of antibody-producing B cells<sup>105,106</sup>. However, the induction of CNS inflammation is inappropriate in the treatment of humans. This provides an unfilled niche in clinical rabies treatment - a therapy which is able to elicit the expression of anti-RABV antibodies behind the BBB early in infection may be able to clear RABV from CNS tissues.

### 1.5.3 Immune evasion

RABV has multiple mechanisms to either evade detection or actively interfere with integral processes of the immune response (Figure 1:8). The accumulation of immune evasion mechanisms employed by RABV proteins result in stark differences between pathogenic and apathogenic RABV infection. Infection with pathogenic RABVs results in diminished activation of the innate immune response and lower APC activation leading to reduced expression of chemokines, cytokines and permeabilisation of the BBB<sup>123</sup>. This in turn leads to a weakened adaptive response which may further be skewed to a Th2 immune response to inhibit effective RABV clearance<sup>118</sup>. Where the adaptive immune response is activated, the lack of BBB permeabilisation further inhibits the ability of immune cells such as macrophages, DCs, T cells, and B cells to migrate into the CNS/brain to clear infectious virus<sup>108,180</sup>. The role of the RABV proteins in immune response evasion are discussed in the following sections.

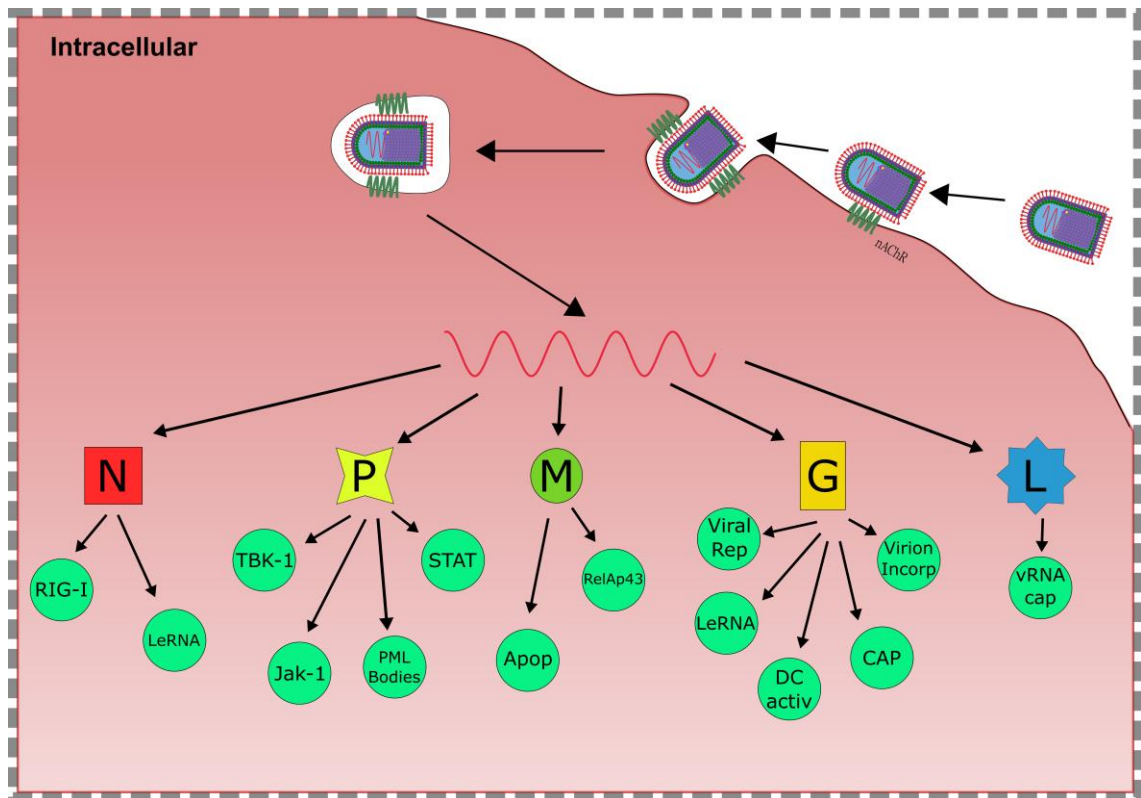


Figure 1:8: Interactions of rabies virus proteins with cells of the infected host to either evade or actively interfere with the activation of the immune system. RIG-I = retinoic acid-inducible gene I, LeRNA = leader RNA, TBK-1 = TANK [TRAF (Tumor Necrosis Factor Receptor–Associated Factor) Family Member Associated NFκB Activator] Binding Kinase 1, Jak-1 = Janus kinase 1, PML bodies = promyelocytic leukaemia bodies, STAT = signal transducer and activator of transcription, Apop = apoptosis, RelAp43= REL-associated protein 43, Viral Rep = viral replication, DC active = dendritic cell activation, CAP = cholinergic anti-inflammatory pathway, Virion Incorp = incorporation into virions.

#### 1.5.3.1 N

Alongside major roles in RABV replication and transcription, the RABV N protein is also able to interfere with the immune response through interactions with LeRNA and IFN induction. LeRNA is produced in large amounts during transcription and is strongly and preferentially bound by unphosphorylated N. This encapsidation of LeRNA by N protein has been demonstrated to significantly reduce binding to viral genomic RNA and, therefore, reduce replication<sup>17,20-23,181,182</sup>. With regards to IFN pathway interference, N protein mediated IFN antagonism has been observed in pathogenic RABV strains. Here, the presence of various mutations (aa 273 and 394<sup>183</sup>) suppress both RIG-I induction and the downstream activation of IRF-3 by changing the RNP structure to prevent RIG-I recognition<sup>181,184</sup>.

### 1.5.3.2 P

The RABV P protein has multiple interactions with the type I IFN pathway. In particular, isoforms 2-5 lack the N-terminal RABV L protein binding domain and have various roles in immune evasion<sup>184</sup>. RABV P protein is able to interfere with the cellular immune response at various stages. In the case of RNA detection by RIG-I, studies have demonstrated that RABV P1 (other isoforms were not explored) is able to inhibit the activation of TANK [TRAF (Tumour Necrosis Factor Receptor–Associated Factor) Family Member Associated NFKB Activator] Binding Kinase 1 (TBK1) to prevent phosphorylation of IRF3<sup>185-187</sup>. P protein isoforms are also able to act downstream in this pathway, where their CTD allows binding to STAT proteins. Here, depending on the localisation of the P isoform, STAT1/2/3 may be actively transported out of the nucleus (P1/2), prevented from entering the nucleus (P3)<sup>184</sup>, or prevented from binding to ISG DNA promoters (P1/3)<sup>188</sup>. Through interactions with RABV M proteins, P protein is also able to partly prevent Janus Kinase-1 (JAK1) phosphorylation, preventing STAT activation and downstream ISG activation<sup>189</sup>. Furthermore, both P1 and P3 have been found to associate with and disrupt promyelocytic leukaemia protein (PML) bodies<sup>35,190</sup>. By influencing IFN-induced PML distribution and structure, P protein is able to effectively attenuate the antiviral effects of PML, including inhibition of viral mRNA transcription, protein synthesis, degradation of activated IRF3, and enhancing IFN $\beta$  production<sup>188,191</sup>.

### 1.5.3.3 M

Rabies M protein, aside from being a potent modulator of cellular apoptosis after RABV infection, is also able to modulate IFN- $\alpha/\beta$  transcription through interactions with NF $\kappa$ B<sup>192,193</sup>. NF $\kappa$ B typically comprises of a p50/RelA or p50/c-Rel dimer bound by an inhibitory I $\kappa$ B protein. In the canonical pathway of activation, I $\kappa$ B is released from the p50/RelA dimer and the p50/RelA dimer is then able to migrate to the nucleus where it is able to promote IFN and inflammatory response gene transcription<sup>193,194</sup>. The RelA splice variant RelAp43 is expressed in human cells and has similar properties to RelA in promoting the transcription of inflammatory genes<sup>193</sup>. The RABV M protein is able to bind RelAp43 and prevent the induction of these genes, effectively limiting the host cell response to infection<sup>192</sup>.

### 1.5.3.4 G

While many proteins within RABV are involved in immune evasion, the rabies G protein is the major activator of the immune response<sup>108,195</sup>. Reverse genetics studies have demonstrated that pathogenic RABV G proteins are able to decrease the rate of viral replication and G protein expression<sup>123,196</sup>. This reduced G protein expression results in the incorporation of significantly (10x) less G protein into newly formed virions<sup>197</sup>. The RABV G, aside from being actively hidden from the immune system, is also a poor activator of DCs. Studies investigating

the infection of DCs with pathogenic and apathogenic RABVs have shown that the former are inferior at infecting, replicating within, and activating DCs. These (ectodomain dependent<sup>123,196</sup>) observations may be attributed to multiple reasons including downregulation of LeRNA production (the cause of DC activation) and inefficient binding to DCs due to deficient incorporation of G protein into virions<sup>197</sup>. Interestingly, pathogenic RABV strains are able to use infected DCs as a vehicle for further infection<sup>129</sup>.

The pathogenic RABV G protein ectodomain is also able to interact with (but not infect) macrophages to promote immune evasion<sup>138</sup>. The binding of RABV to the nAChR receptor on macrophages has demonstrated the induction of the cholinergic anti-inflammatory pathway (CAP). In the context of RABV infection, activation of the CAP was shown to cause macrophage cytoplasmic NFκB retention, increasing anti-inflammatory IL-10 production, decreasing CD4<sup>+</sup> T cell activation, and long-term exposure causing a shift to anti-inflammatory macrophage M2-c phenotype<sup>138</sup>.

#### 1.5.3.5 L

Lastly, the RABV L protein also plays a major role in immune evasion, mediated largely through the C-terminal lysine-aspartic acid-lysine-glutamic acid (KDKE) motif. Originally characterised in VSV, this highly conserved motif is heavily involved in vRNA capping (imitates a MTase), which is essential for vRNA translation. In the absence of MTase activity, vRNA may activate MDA5 (dsRNA sensor which promotes IFN production<sup>198</sup>) or interferon-induced protein with tetratricopeptide repeats-1/2 (IFIT-1/2) (restriction factor able to block replication of RABV in the cytosol<sup>199,200</sup>). However, upon *in vitro* investigation, the mutation of the two lysine (K) sites had no effect on IFN production (suggesting that MTase activity has little involvement with RIG-I/MDA5 recognition) but overexpression of IFIT2 inhibited RABV replication by 60%. Subsequent *in vivo* investigation also revealed attenuated pathogenicity upon mouse infection, with mice showing no clinical signs of infection. Two hypotheses were established, either the KDKE motif is extensively involved in RABV replication, or the lack of MTase activity enables increased sensitivity to IFIT2<sup>198</sup>.

## 1.6 PRE- AND POST-EXPOSURE RABIES MANAGEMENT

In the management of rabies, both Pre-exposure prophylaxis (PrEP) and PEP are utilised. PrEP consists entirely of vaccination and is recommended for laboratory scientists, travellers, animal handlers and other individuals at high risk of RABV exposure. PEP, meanwhile, consists of three parts depending on the severity of RABV exposure. These include the appropriate washing of the wound, vaccination, and the administration of rabies immunoglobulin (RIG). How, where, and when these treatments are administered depends on the chosen PEP regimen, as multiple

are available. RIG may be either human (HRIG) or equine (ERIG)-derived and is administered both in and around the wound <sup>201</sup>. Furthermore, vaccination alone displays reduced effectiveness as PEP unless combined with RIG <sup>202</sup>. However, while PEP is available there are restrictions around its utilisation; 1) PEP is only effective if given before the onset of clinical disease <sup>203</sup>; 2) PEP is often not immediately available in endemic areas; 3) A lack of awareness of rabies virus and the fatal disease it causes can mean that PEP is not sought following potential exposure. Furthermore, costs associated with vaccine and RIG can be prohibitively high. Thus, despite the lower cost of ERIG (\$25-50 /dose) compared to HRIG (\$100-250 /dose), ERIG is still unaffordable in many countries <sup>201,204,205</sup>. Fortunately, recent advances in anti-RABV monoclonal antibodies (mAb) have resulted in the World Health Organisation (WHO) recommending them as RIG alternatives where available <sup>206</sup>.

### 1.6.1 Pre-exposure prophylaxis

Inactivated rabies vaccines for human use have, since the development of cell culture-derived preparations, remained unchanged for decades. Inactivated vaccines include cultivating an attenuated virus, such as CVS-11, Pasteur virus, Pittman-Moore-NIL2, or Nishigahara strain <sup>207</sup>, before inactivating them (most commonly) with beta propiolactone (BPL). Historically, these viral strains were grown in nerve tissue, however, due to a range of side effects associated with nerve tissue derived vaccines, in 2005 the WHO officially recommended the discontinuation of their use (although some countries including Bolivia, Algeria, Ethiopia, and Argentina are reported to still use nerve tissue derived vaccines <sup>208</sup>). Now, all inactivated vaccines are propagated in either cell culture or avian embryos (Table 1:1).

|                                                 |                                                                                                         |
|-------------------------------------------------|---------------------------------------------------------------------------------------------------------|
| Cell culture and embryonated egg-based vaccines | <u>HDCV</u> – HEP Flury strain, grown in MRC-5 diploid cell culture, inactivated by BPL                 |
|                                                 | <u>PCECV</u> – HEP Flury strain, grow in chick fibroblasts, inactivated by BPL                          |
|                                                 | <u>PVRV</u> – Wistar strain, grown in Vero cell fermenters, inactivated by BPL                          |
|                                                 | <u>PHKCV</u> – Beijing strain, grown in hamster kidney cells, inactivated by formalin                   |
|                                                 | <u>PDEV</u> – grown in duck embryos, inactivated by $\beta$ -propiolactone                              |
| Nerve Tissue Vaccines                           | <u>Semple vaccine</u> - BPL or phenol inactivated virus from infected goat/sheep brain tissue           |
|                                                 | <u>Fuenzalida vaccine</u> – Ultraviolet (UV) light inactivated virus from infected suckling mouse brain |

Table 1:1: Current and historical types of rabies vaccines. Rabies vaccines can be separated into two groups; Cell culture and embryonated egg-based (CCEEVs); or Nerve tissue vaccines (NT). HDCV: Human diploid cell vaccine. PCECV: Purified chick embryo cell vaccine. PVRV: Purified Vero cell rabies vaccine. PHKCV: Primary hamster kidney cell vaccine. PDEV: Purified duck embryo vaccine.

Such vaccines are safe, highly immunogenic, and induce sufficient neutralising antibody profiles, although multiple clinic visits are required to provide long-lasting protection in line with WHO vaccination recommendations [serum neutralisation test giving a titre of at least 0.5 international units (IU) per millilitre (mL)]<sup>209-211</sup>. To counter this, optimised regimens have been developed to reduce vaccine use and ensure available supplies are utilised most appropriately<sup>1,205,209,212,213</sup>.

However, despite their utility in pre- and post-exposure immunisation, inactivated rabies vaccines are unable to prevent rabies replication following clinical disease onset<sup>105,214-218</sup>. Although inactivated vaccines have been shown to induce serum antibody titres post-vaccination, they do not induce BBB permeabilisation<sup>219</sup>. Therefore, with the BBB's molecular exclusion limit for molecules >400 Da<sup>88</sup>, few antibodies and immune cells are able to pass through the BBB and counter virus replication in the CNS<sup>94</sup>. Transient opening of the BBB is known to occur when inflammatory responses are triggered in the CNS<sup>220</sup>, however, while this has successfully promoted viral clearance in a mouse strain more susceptible to BBB permeability changes in response to infection<sup>106</sup>, inducing CNS inflammation during human infection is likely counterproductive due to associated pathologies (stroke, epilepsy, depression, and anxiety)<sup>221</sup>.

#### 1.6.1.1 PrEP Vaccines - Current research into live viruses and recombinants

Despite the safety and immunogenicity (accompanied with the ability to induce neutralising antibody profiles) of inactivated RABV vaccines, they are unable to prevent RABV replication following clinical disease onset<sup>105,214-218</sup>. While inactivated vaccines are unable to promote adequate BBB permeability to enable sufficient CNS immune infiltrate. Advances in the development of live attenuated virus vaccines (LAVVs) have improved opportunities to utilize these vectors to deliver effector molecules to the CNS. The immune response elicited in the CNS following vaccination with a live-attenuated virus is not associated with any significant pathology<sup>105</sup> and live-attenuated RABV vaccines have consistently been shown in experimental models to facilitate both an increase in BBB permeability and to protect against or clear lethal virus infection<sup>214,215</sup>. These features of LAVVs support their potential for therapeutic use. Certainly, a LAVV that can effectively increase the permeability of the BBB and promote sufficient immune cell infiltration to clear virus infection during the clinical stages of disease is a high priority for rabies post-infection treatment (PIT) research.

Historically, the first successful LAVV was used in the eradication of smallpox in 1980. Since then, various LAVVs have been developed to combat diseases such as poliovirus, yellow fever virus, rinderpest virus, measles virus, and mumps virus<sup>222</sup>. Live attenuated rabies vaccines are

currently only used for the protection of animals using a liquid virus containing sachet incorporated into the animal's bait <sup>207</sup>, as intact virus either replicating locally in the oral mucosae or following ingestion and exposure in the intestinal tract is sufficient to cause an immune response and subsequent protection <sup>223,224</sup>. The attenuated strains used in the live vaccination of animals are descended from the parental Street Alabama Dufferin (SAD)-Bern strain (a derivative of the SAD strain) <sup>225</sup>. These include SAD B19, SAD Bern, SAD P5/88, SAD Avirulent Gif (SAG) 1/2, SAD VA1, V-RG, ERA G333, KMIEV-94 and RV 97 <sup>225-227</sup>.

#### 1.6.1.1.1 Characteristics of attenuated viruses

In *in vivo* studies, pathogenic and apathogenic RABV strains are easily distinguished. Pathogenic RABV strains, upon peripheral infection, cause mortality while apathogenic strains do not. Regarding differences seen *in vitro*, pathogenic and apathogenic viruses can be differentiated in three aspects: viral growth kinetics, time taken to infect a cell, and ability to spread between cells <sup>228,229</sup>. While there are exceptions <sup>230</sup>, pathogenic RABVs generally replicate less efficiently (reach lower maximum titres in growth curves) but infect and spread between cells quicker <sup>228,229</sup>. When taken into consideration alongside the benefits of avoiding the immune response, these observations are unsurprising. Apathogenic RABVs, by definition, are easily detected by the host immune system. The combination of rapid replication with the diminished capacity to infect and spread between cells means apathogenic RABV is exposed extracellularly to the immune system for extended periods of time. In contrast, the slow replication rate of pathogenic RABVs means that a significantly smaller numbers of RABV virions are released into the extracellular medium. Furthermore, any RABVs that are released are able to quickly infect neighbouring cells resulting in a lower exposure time to the host immune system, decreasing the ability of the host to elicit an immune response.

#### 1.6.1.1.2 Methods of RABV attenuation

Currently, three methods have been investigated for the attenuation of pathogenic RABV strains. Firstly, reverse genetics may engineer specific site mutations, insertions, or deletions within the RABV genome. Secondly, the order of RABV genes may be either rearranged, or individual genes duplicated or exchanged. Thirdly, non-viral elements (NVEs) may be inserted within the RABV genome.

##### 1.6.1.1.2.1 Attenuation by Mutation

The RABV G protein has received substantial attention in research focusing on pathogenic attenuation by mutation. Multiple mutational events have been demonstrated to affect RABV pathogenicity. Firstly, and most recently, a glycine (Gly) to aspartic acid (Asp) mutation at position 255 was shown to significantly decrease neurotropism and slow viral spread and

growth<sup>230</sup>. Secondly, a Gly to glutamic acid (Glu) mutation at position 349 has also been demonstrated to attenuate viral pathogenicity<sup>231</sup>. However, in contrast to the Asp255Gly mutation<sup>230</sup>, the Gly349 mutation was not reported to affect neurotropism and viral spread/growth. Instead, a Gly349Glu mutation attenuated RABV through the enhanced activation of the immune system<sup>231</sup>. Thirdly, mutation of aa 83 from K to arginine (R) has been demonstrated to significantly decrease RABV pathogenicity through the increased expression of RABV and TJ-degrading matrix metalloproteinase<sup>232</sup>. Fourth, analysis of naturally occurring RABV strains has revealed that both an R to isoleucine (Ile) or Ser mutation at positions 196 were able to elicit enhanced viral production through additional N-terminal G protein glycosylation<sup>233</sup>.

Mutation of aa 333 and 194 have been the focus of a large amount of research and resulting attenuated viruses have been extensively used in the vaccination of wildlife<sup>234-236</sup>. R333 is located in G proteins AS-III, a conformational epitope linked to viral pathogenicity and neuroinvasiveness common across all Lyssaviruses<sup>235</sup>. By mutating R to Glu, viral pathogenicity has been effectively attenuated<sup>235</sup>. However, due to a high mutation rate, escape mutants were described after just five passages in suckling mice, where a rabies vector containing the R333Glu (SPBNG) substitution reverted back to a pathogenic phenotype after a single asparagine (Asn) to K mutation at residue 194<sup>228</sup>. However, pre-emptive mutation of 194 from Asn to Ser was able to abolish reversion to pathogenicity<sup>228</sup>. This attenuated G gene has since been duplicated and triplicated within a pSPBN vector (developed from the SAD strain) for further attenuation<sup>236-239</sup>. This was effective in increasing expression of the G protein, resulting in higher VNA titres, permeabilisation of the BBB, and infiltration of immune cells (preferentially B cells) into the CNS<sup>108,188,237-241</sup>.

Additional attenuating mutations have been introduced in RABV genes including N and P. These were discovered either by the analysis of attenuated strains of pathogenic viruses or through the investigation of RABV protein function. Two N protein mutations, phenylalanine (Phe) to leucine (Leu) at position 273 and tyrosine (Tyr) to histidine (His) at position 394 were discovered to be the cause of Ni-CE RABV strain apathogenicity (compared to pathogenic Ni strain). These mutations promoted RIG-I activation and strongly decreased pathogenicity<sup>217,242,243</sup>. Studies investigating the mutation of the LC8 binding region within RABV P have also resulted in the discovery of a novel method of attenuation. While abolishing the LC8 binding region of P alone has little effect on virus pathogenicity in suckling mice, combination with a RABV G protein aa 333 mutation significantly reduced pathogenicity<sup>244</sup>.

#### 1.6.1.1.2.2 Insertion of non-viral elements

As described, *in vivo* infection with attenuated RABV causes a wide range of cytokine and chemokine upregulation, with functions ranging from upregulating MHC expression to an increase in BBB permeability<sup>123,245</sup>. While some vaccines aim to only activate the immune system through the presentation of attenuated G proteins, there has recently been a focus on the expression of non-viral elements as a way of supplementing or proactively stimulating different elements of the host immune response. A number of studies have since addressed the incorporation of immune stimulatory or regulatory molecules (some of which have been highlighted for their role in BBB permeabilisation<sup>93,245-247</sup>) into the rabies genome, with the majority of novel recombinant vaccines demonstrating over 90% protection. Following studies into post-vaccination immunological responses and to further interrogate the effects of non-viral elements post-infection, a number of challenge studies have been reported. These include; macrophage inflammatory protein 1 $\alpha$  (MIP-1 $\alpha$ ), granulocyte macrophage colony stimulating factor (GM-CSF), macrophage-derived chemokine (MDC), flagellin, high mobility group box protein 1 (HMGB1), DCBp, CXCL13, cytochrome c, common  $\gamma$ -chain cytokine family members IL-15, IL-21, IL-6, IL-18, and IL-7, TNF- $\alpha$ , IFN $\alpha$ 1, IFN $\gamma$ , and OX40-Ligand (Table 1:2)<sup>218,248-261</sup>. The majority of these elements have been cloned into the vaccine strain LBNSE, between the G and L genes, while some (DCB<sub>p</sub>) have been inserted within the G protein itself<sup>256</sup>.

| <u>Non-viral element</u>                    | <u>Function</u>                                                                                                                                                                                 | <u>Produced by</u>                                                                   |                                                  | <u>Effect</u>                                                                                       |
|---------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|--------------------------------------------------|-----------------------------------------------------------------------------------------------------|
| MIP-1 $\alpha$ /CCL3*<br><sup>262-264</sup> | Inflammatory chemokine, promotes and activates monocyte/macrophage /neutrophils, and DC-progenitor cells.                                                                                       | Lymphocytes, fibroblasts and epithelial cells                                        | Increased survivorship (VNA mediated protection) | Local cytokine production increase, T/B cell recruitment, DC/B cell activation <sup>265</sup>       |
| GM-CSF<br><sup>266</sup>                    | Pro-inflammatory cytokine. Aids the development of DCs and recruits/enhances myeloid cell survival and activation.                                                                              | Fibroblasts, endothelial cells, macrophages, DCs, T cells, neutrophils, eosinophils. |                                                  | Maturation and activation of BMDCs, recruitment/activation of DC/B/T cells <sup>265</sup>           |
| MDC/CC L22<br><sup>267</sup>                | Migration of activated T, Treg, NK cells, monocytes, and monocyte derived DCs                                                                                                                   | Macrophages, monocyte-derived DC's, and activated NK / T cells, keratinocytes.       |                                                  | DC/B cell activation/maturation <sup>248</sup>                                                      |
| Flagellin<br><sup>268</sup>                 | Filamentous bacterial protein. Induces the migration and maturation of DC and neutrophils.                                                                                                      | Bacteria                                                                             |                                                  | Increased apoptosis, development of N protein inclusion bodies in neuronal cell body <sup>260</sup> |
| Cytochrome c<br><sup>269</sup>              | Transmembrane protein involved in the electron transport from complex III to IV within the electron transport chain. Upon membrane release has roles in caspase-dependent apoptosis initiation. | All cells undergoing cellular respiration                                            |                                                  |                                                                                                     |

|                                            |                                                                                                                                                                                          |                                                                                                                  |                                                  |                                                                                                                                                                            |
|--------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------|--------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| CXCL13<br>270-272                          | Promotes B and Tfh cell recruitment to B cell follicles.                                                                                                                                 | Stromal cells, follicular DC (FDC), Tfh cells, mature B cells.                                                   |                                                  | Tfh/GC B cell recruitment, GC formation <sup>249</sup>                                                                                                                     |
| IL-21 <sup>273-275</sup>                   | Activation and differentiation of NK, NK-T, B, and CD8 cells.                                                                                                                            | T and NK T cells                                                                                                 |                                                  | Generation of activated DCs/Tfh/GC B cells <sup>251</sup>                                                                                                                  |
| IL-15 <sup>276-279</sup>                   | Similar functions to IL-21 in the development of T, B, NK, and NK T cells                                                                                                                | Monocytes, macrophages, and DC's.                                                                                |                                                  | Increased generation of Tfh/GC B/plasma cells and DC maturation <sup>250</sup>                                                                                             |
| IL-7 * <sup>252</sup>                      | Proliferation and maintenance of T/B cells, generation of Tfh and GC B cells, and promotes survival of CD4/8 T cells                                                                     | Stromal cells present in adult bone marrow                                                                       |                                                  | Increased GC B cell presence in inguinal lymph nodes, increased Tfh population <sup>252</sup>                                                                              |
| IL-18 <sup>280</sup>                       | Pro-inflammatory cytokine able to induce IFN $\gamma$ in a variety of immune cells (CD4/8, NK cells, and macrophages), activates NK cells, and upregulates cytotoxicity in NK/CD8 cells. | Monocytes, macrophages, DC's, endothelial cells, keratinocytes and intestinal epithelial cells                   |                                                  | Increased levels of CD4/8 T cells, IFN $\gamma$ -secreting CD4/8 T cells, and activated B cells in blood/lymph nodes <sup>258</sup>                                        |
| IL-6 * <sup>257</sup>                      | Stimulates PC development and T cell proliferation/development, with major roles in mucosal IgA antibody responses                                                                       | Various cell types, primary sources being monocytes and macrophages                                              |                                                  | Increased BBB permeability, recruitment of B/T cells to lymph nodes, and chemokine production in CNS <sup>257</sup>                                                        |
| OX40L <sup>261</sup>                       | Involved in the generation of Tfh and GC cells, promoting development of high-affinity antibody secreting PCs                                                                            | Activated T and B cells, mast cells, vascular endothelial cells, and APCs (macrophages and DCs)                  |                                                  | Large number of GC and B cells generated, associated with prolonged high-affinity (IgG2a and IgG2b) protective antibody response <sup>261</sup>                            |
| TNF- $\alpha$ * <sup>281-284</sup>         | A pro-inflammatory cytokine expressed as either a soluble or transmembrane protein with roles in cellular differentiation, proliferation, and death.                                     | TNF-R1 is expressed in most tissues, while TNF-R2 is found in immune system cells.                               |                                                  | T cell infiltration to brain tissue and increased reactive microgliosis <sup>253</sup>                                                                                     |
| IFN $\gamma$ * <sup>285,286</sup>          | A pleiotropic cytokine involved in Th1 immune response, including the activation of macrophages and the production of chemokines.                                                        | Initially expressed by NK and NK-T cells. Then later by CD4/8 cells.                                             |                                                  | Increased survivorship (not VNA mediated), no increase in BBB permeability, type I IFN induction <sup>254</sup>                                                            |
| IFN1 $\alpha$ /IFN $\alpha$ <sup>287</sup> | A type 1 IFN, able to induce antimicrobial states within neighbouring cells, module innate immune responses for antigen presentation, and promote immunological memory.                  | Predominantly haematopoietic cells, especially plasmacytoid DC cells                                             | Increased survivorship (VNA mediated protection) | Stronger innate immune response, no increased apoptosis, increased levels of activated B and CD8 T cells in lymph nodes and peripheral blood respectively <sup>259</sup> . |
| HMGB1 <sup>255,288</sup>                   | Usually intracellular but may act as a pro-inflammatory cytokine upon release, promoting DC maturation, activation, and migration into lymph nodes.                                      | Actively secreted by DCs, NK cells, monocytes, and macrophages. Passively secreted by necrotic or damaged cells. |                                                  | Increased Tfh/GC B/plasma cell recruitment and activation <sup>255</sup>                                                                                                   |
| DCB <sub>p</sub> <sup>256,289</sup>        | N/A                                                                                                                                                                                      | N/A – Extracted from a peptide library                                                                           |                                                  | Increased DC binding/activation and Tfh/GC B cell generation <sup>256</sup> .                                                                                              |

Table 1:2: A list of the various non-viral elements have been incorporated into the genome of LAVVs (Live Attenuated Virus Vaccines). Those marked with an asterisk (\*) are those which have been previously suggested as molecules which are involved in the permeabilisation of the BBB. MIP-1 $\alpha$ =Macrophage inflammatory protein  $\alpha$ . CCL3=Chemokine (C-C motif) ligand 3. GM-CSF=Granulocyte-macrophage colony-stimulating factor. BMDC=Bone marrow derived dendritic cell MDC=Macrophage-derived chemokine. CCL22=Chemokine (C-C motif) ligand-22. NK=Natural killer. CXCL13=chemokine (C-X-C motif) ligand-13. Tfh=T follicular helper cell. GC B=Germinal centre B cells. IL-21=Interleukin-21. IL-15=Interleukin-15. IL-2=Interleukin-2. PC=Plasma cell. IL-6=Interleukin-6. TNF- $\alpha$ =Tumour necrosis factor- $\alpha$ . IFN- $\gamma$ =Interferon- $\gamma$ .

## 1.6.2 Post-exposure treatments and prophylaxis

### 1.6.2.1 Milwaukee protocol

Rabies, upon the onset of clinical symptoms, is traditionally regarded as an incurable disease<sup>203</sup>. However, pre- and post-exposure prophylaxis therapies, when administered appropriately and in a timely manner, are highly effective in preventing the onset of disease<sup>290,291</sup>. While cases of true PEP failure (where PEP is administered completely and appropriately) do exist<sup>292-294</sup>, the majority of instances of failure can be attributed to errors in administration<sup>294,295</sup> or possible direct infection of RABV into the CNS or other neural tissues<sup>71</sup>. In 2005, an experimental treatment was performed on a symptomatic rabies-infected patient from Wisconsin which was later termed the “Milwaukee Protocol”. This protocol combined anti-excitatory and antiviral drugs, including ketamine, ribavirin, and amantadine, in conjunction with supportive intensive care<sup>296</sup>. Here, the patient was not given either rabies vaccine or RIG due to the patient's VNA response and the theoretical potential for harm from an altered immune response. After >70 days of hospitalization, the patient recovered with only minor neurologic sequelae and became the first documented case of a patient to survive clinical rabies without prior history of vaccination<sup>297</sup>. The Milwaukee protocol has since been used to treat a small number of patients worldwide. However, due to lack of success and supporting scientific evidence behind multiple aspects of the treatment, the Milwaukee protocol is no longer considered a therapeutic application for treating rabies in humans<sup>298-300</sup>.

### 1.6.2.2 Monoclonal antibodies

#### 1.6.2.2.1 62-71-3

In 2002, the WHO proposed 6 RABV-neutralising monoclonal antibodies (mAbs) for investigation: 62-71-3, E559, 1112-1, D8, M777, and M727<sup>301-303</sup>. Of these (after the exclusion of 1112-1 and mAb D8 due to intellectual property conflicts and insufficient strain coverage, respectively), all targeted AS-II, with the exception of 62-71-3 which targets AS-I (with epitope

mapping revealing specificity to aa residue K226 in the G protein)<sup>302,304,305</sup>. 62-71-3 was expressed both as a single chain fragment variable molecule (ScFv) in a phage display system to investigate neutralisation and a chimeric murine-human antibody, which was later expressed in a ΔXF (glycosylation mutant lacking the ability for fucose and xylose glycosylation<sup>306</sup>) *Nicotiana benthamiana* plant expression system<sup>302,307</sup>. These were shown to be effective at neutralising phylogroup I Lyssaviruses (such as RABV), but not phylogroup II or III Lyssaviruses.

Despite their small size, ScFvs are unable to cross the BBB and infiltrate the CNS in high enough concentrations to neutralise CNS-localised RABVs. To overcome this shortcoming, a plant-produced RABV glycoprotein peptide (RVGp)-conjugated 62-71-3 ScFv was engineered. It was hypothesised that the RVGp, a small 29 aa peptide specific for cell receptor binding and entry, would allow the ScFv to copy the natural route of RABV infection, allowing access to the CNS. Initially, the entire ScFv- RVGp fusion protein was expressed in plants and equivalent RABV neutralisation data between ScFv and ScFv-RVGp indicated that neutralisation was not impaired by RVGp fusion<sup>308</sup>.

To investigate whether this fusion protein could cross the BBB, an *in cellulo* BBB model was used in which hCMEC/D3 (human cortical microvessel endothelial cells) cells expressed nAChR. Upon addition, full-length 62-71-3 mAb with or without RVGp conjugation did not cross the monolayer, a small amount of non-RVGp fused ScFv did cross whilst the RVGp-fused ScFv was shown to be actively transported across the monolayer as early as 2 hours (h) after addition to the *in vitro* model<sup>302,308</sup>.

Following these findings, the 62-71-3 ScFv RVGp fusion protein was then studied *in vivo*. However, due to low expression in the plant system, 62-71-3 ScFv was chemically conjugated to an RVGp, rather than expressed as a fusion protein (this did not affect ability to neutralise RABV). To assess the effectiveness of 62-71-3 ScFv/RVGp against RABV challenge, C57BL/6 mice were intraperitoneally (ip) treated either four h before or two to four days after im CVS (challenge virus standard) challenge (105.8 TCID<sub>50</sub>/mL). 62-71-3 ScFv/RVG was compared against HRIG, the anti-viral T705 (favipiravir), and PBS. While ScFv/RVG treatment halved the mortality rate relative to the PBS control, it could not match the survival rate of mice treated with HRIG. However, when combined with T705, survival rates were comparable<sup>309</sup>. As discussed within the study, these results do not necessarily indicate that 62-71-3 ScFv was ineffective (compared to HRIG) for rabies treatment, rather, it may be the differing pharmacokinetics between ScFvs and RIG antibodies (ScFvs have a shorter half-life) that prevented 62-71-3 ScFv from preventing mortality to levels equivalent to HRIG<sup>309</sup>.

#### 1.6.2.2.2 The successful treatment of clinical rabies in mice

Since the WHO recommendation of five monoclonal antibodies for inclusion into antibody cocktails for the PEP treatment of rabies <sup>301,303</sup> multiple other anti-RABV mAbs have been discovered. One prominent example is the mAb cocktail of RVC20 and RVC58 which, importantly, was able to cure mice of symptomatic rabies infection <sup>310</sup>. RVC20 and RVC58, bind different antigenic sites of the RABV G protein (I and III, respectively) and were shown to neutralise almost all phylogroup I Lyssaviruses tested and some Lyssaviruses from phylogroup II and III <sup>311</sup>. By combining RVC20 and RVC58 into a single cocktail (1:1 mixture), this was able to neutralise 100% of phylogroup I Lyssaviruses tested (HRIG was only able to neutralise 38%) <sup>311</sup>. First, the RVC20/RVC58 cocktail was assessed in the ability to prevent rabies onset if administered im. Two doses were selected, 2 and 20 milligram (mg) /kilogram (kg), and while these were effective at early timepoints, 20 mg/kg was only able to protect 20% of mice when delivered 6 dpi. As this suggested peripheral RVC20/RVC58 cocktail administration was unable to prevent mortality, mice were fitted with an intracerebroventricular (ICV) pump to deliver mAb cocktail infusions alongside a single IM dose, starting at six, seven, or eight days after lethal challenge. These timepoints were selected as, within this study, RABV was thought to already have migrated to the CNS, was impacting motor performance, or mice were exhibiting classical signs of murine RABV infection. The regimes explored within this study were able to prevent mortality in 100% of mice when begun on day six (virus thought to be present in the CNS) and 55% of mice when treatment began on day seven (slight affected motor performance). Most importantly, when treatment began after the onset of clinical signs, 33% of mice recovered from infection.

The results from this study <sup>310</sup>, demonstrate the importance of antibody expression within the brain for the treatment of clinical rabies infection. While the use of an ICV pump was evidently effective, the deaths of three mice during ICV infusion demonstrate the risks of such an invasive procedure <sup>310</sup> and indicate the need for a less-invasive method of delivering or eliciting high levels of RABV-neutralising antibody expression within the brain/CNS.

#### 1.6.3 Concluding statements

Since the development of the Milwaukee protocol (and the similar Recife protocol <sup>298</sup>) no major novel rabies treatment regimens or therapies have been approved for use in humans. However, as discussed previously, notable scientific advances have been made using LAVVs <sup>180</sup> and mAbs <sup>310</sup> in studies *in vivo*. As has been reviewed previously, in the context of post-symptomatic treatment, a large proportion of research has recently focused upon the attenuation of RABV by the insertion of NVEs or multiplication of attenuated RABV G genes <sup>238,239</sup>. These LAVVs have consistently been able to promote strong migration of T cells, B cells,

and APCs into the brain and CNS. Where assessed, this has been accompanied by the permeabilisation of the BBB <sup>180</sup>. While the majority of novel LAVVs have been assessed as PrEP vaccination alternatives, some have been evaluated in their ability to prevent mortality if given as a post-infection treatment. LAVVs that have been investigated in this regard include TriGAS (also known as SPBAANGAS-GAS-GAS) <sup>238</sup>, SRV9 <sup>312,313</sup>, or a GM-CSF expressing LBNSE rabies virus vector <sup>314</sup>. These were all effective in preventing mortality in a minimum of 20% of mice lethally challenged peripherally with rabies virus. While these studies have not investigated the effectiveness of LAVV administration as a post-symptom onset treatment (only up to six days post-infection), they indicate that LAVVs represent a possible method for the post-clinical onset treatment of rabies. Another method, although significantly more invasive than LAVV administration, is the ICV administration of mAb cocktails directly onto the brain of lethally infected mice. This has, as discussed, been effective in successfully curing symptomatic mice of RABV infection <sup>310</sup>. This represents the greatest step towards true symptomatic treatment of rabies. However, this technique is both highly invasive, expensive, intensive, relies on large volumes of mAb, and the procedure alone caused the death of multiple mice.

## 1.7 PROJECT OUTLINE

This thesis will focus on the development of a novel post-infection treatment (PIT) for rabies. The hypothesis of this thesis is that a RABV-based LAVV which expresses a RABV-neutralising protein is able to replicate within the host and cause a robust immune response within the CNS to deliver high quantities of RABV neutralising antibodies into the brain/CNS to cure mice of symptomatic rabies. Specifically, this novel PIT will use a RABV-based LAVV to express a fluorescent secreted neutralising anti-RABV scFv molecule. By using a RABV-based LAVV, neutralising scFv proteins will be produced both in high concentrations (due to the ability of the LAVV to replicate in the host) and behind the BBB as the LAVV follows typical route of RABV infection. Furthermore, this LAVV will also activate the host immune system which will result in the clearance of both itself and any lethal RABVs present.

The objectives of this thesis are as follows

- Successfully design and rescue a scFv-expressing rabies LAVV.
- Through *in vitro* analysis, assess the toxicity of this virus upon cellular infection, alongside viral growth and dissemination kinetics, and the ability to produce neutralising scFv protein.
- Assess the safety of this scFv-expressing rabies LAVV in a mouse model, evaluating different doses and routes of inoculation.
- If the LAVV is shown to be safe in a mouse model, assess its effectiveness in preventing the onset of disease or mortality when given either before or after the onset of symptoms, respectively.

## Chapter 2 - METHODS AND MATERIALS

### 2.1 STANDARD BUFFERS AND SOLUTIONS

Details of all buffers and solutions used in this thesis have been described in Appendix 1.

### 2.2 OLIGONUCLEOTIDE PRIMERS AND ENZYMES

#### **Oligonucleotide Primers**

Oligonucleotide primers were purchased from either Merck or EuroFins Genomic.

Oligonucleotide primers purchased from Merck were ordered using the following specifications: primers were synthesised at 0.05 micromole ( $\mu$ mole), purified by desalting, and suspended in water at 100 micromolar ( $\mu$ M). Primers used for Q5<sup>®</sup> Site-Directed Mutagenesis (Section 2.9) were high-performance liquid chromatography (HPLC) purified. Where primers were purchased from EuroFins Genomic, Salt Free Oligo primers were purchased at 0.01  $\mu$ mole and purified by desalting.

All primers upon delivery were diluted 1/10 with distilled water to 10  $\mu$ M concentration. All oligonucleotide primers were stored at -20 °C.

#### **Restriction enzymes**

Restriction enzymes were purchased from New England Biolabs and were used and stored according to the manufacturer's instructions.

#### **Q5<sup>®</sup> High-Fidelity DNA Polymerase**

The New England Biolabs (NEB) Q5<sup>®</sup> DNA Polymerase kit (M0492L) was purchased from New England Biolabs and stored at -20 °C.

#### **GoTaq<sup>®</sup> Green Master Mix**

The Promega GoTaq<sup>®</sup> Green Master Mix kit (M7122) was purchased from Promega and was stored at -20 °C.

#### **NEBuilder<sup>®</sup> HiFi DNA Assembly**

The NEBuilder<sup>®</sup> HiFi DNA Assembly Cloning Kit (E5520S) was purchased from New England Biolabs and stored at -20 °C.

## QIAGEN OneStep RT-PCR Kit

The QIAGEN OneStep RT-PCR [reverse transcription polymerase chain reaction (PCR)] Kit (210210) was purchased from Qiagen and was stored at -20 °C

## 2.3 MAINTENANCE AND CULTURE OF MAMMALIAN CELL, VIRAL, AND BACTERIAL STOCKS

### 2.3.1 Maintenance of mammalian cell lines

Baby hamster kidney 21 (BHK-21), BSR-T7/5 (a BHK-21 cell line derivative), human embryonic kidney 293T (HEK-293T), neuronal-2a (N2a), and Chinese hamster ovary K1 (CHO-K1) cell lines were maintained in T75 flasks at 37 °C 5% CO<sub>2</sub> and passaged upon reaching 80-100% confluency. To passage cell lines, all media was removed and cells briefly washed with three mL of antibiotic-trypsin-versene (ATV) (Section 2.1). Cells were then incubated with fresh ATV until cells disassociated from the bottom of the flask, where fresh media was added to a final volume of 10 mL. Cells were then resuspended by gently pipetting, and one to four mL added to a fresh flask to a final volume of 20 mL. Table 2:1 gives describes the growth media, foetal bovine serum (FBS) concentration, and penicillin-streptomycin (pen-strep) concentration used in these cell lines.

| Cell Line | Media                      | FBS Concentration | Antibiotic concentration                     |
|-----------|----------------------------|-------------------|----------------------------------------------|
| BHK-21    | GMEM                       | 5-10%             | 10 U /mL penicillin<br>10 µg/mL streptomycin |
| BSR-T7/5  | GMEM                       |                   |                                              |
| HEK-293T  | DMEM                       |                   |                                              |
| N2a       | DMEM                       |                   |                                              |
| CHO-K1    | EMEM                       |                   |                                              |
| hCMEC/D3  | EndoGRO™-MV Complete Media | n/a               | n/a                                          |

Table 2:1: Cell culture media used in the maintenance of the different mammalian cell lines used in this thesis. U: units, µg: microgram, GMEM: Glasgow Minimum Essential Medium, DMEM: Dulbecco's Modified Eagle Medium, EMEM; Eagle's minimum essential medium.

### 2.3.2 hCMEC/D3 cells

hCMEC/D3 cells were purchased from Merck (SCC066) and maintained according to the manufacturer's instructions.

#### Extracellular matrix (ECM) coating of T75 flasks

Collagen Type I rat tail (Merck, 08-115) was diluted 1/20 with PBS and used to coat a T75 flask (5-10 mL needed). The flask was then incubated for one h. Before use, the coating solution was aspirated.

#### Preparation of EndoGRO™-MV growth medium.

As repeated thawing to 37 °C may result in degradation of some of the components in complete EndoGRO™-MV growth medium, media was made as required and stored for only two weeks. After thawing, all components were added to EndoGRO™ Basal Medium to the following specifications.

0.2 % EndoGRO-LS supplement

5 ng/mL rh EGF

10 millimolar (mM) L-Glutamine

1 microgram (µg) /mL Hydrocortisone Hemisuccinate

0.75 Units (U) /mL Heparin Sulphate

50 µg/mL Ascorbic Acid

5 % FBS

#### Subculture of hCMEC/D3 cells

After removal of growth medium from T75 flasks, five mL of ATV was added and incubated at 37 °C for three to five minutes (min). After complete detachment of cells, eight mL of pre-warmed (37 °C) complete EndoGRO™-MV growth medium was added and cells transferred to a 50 mL falcon tube. Cells were then centrifuged at 300 x g for 3-5 min and supernatant discarded. hCMEC/D3 cell pellet was resuspended thoroughly in 2 mL of pre-warmed hCMEC/D3 media and the desired dilution (1/3 to 1/6 in complete EndoGRO™-MV growth medium) of cells added to a fresh pre-coated T75 flask.

#### **2.3.3 Storage of Viral Stocks**

Viral stocks were stored in 0.5 mL aliquots in two mL O-Ring screw cap tubes at -80 °C. Viral stocks were either stored solely as tissue culture supernatant, or as a mix of tissue culture supernatant and cell lysate (after centrifugation and removal of cell debris).

#### **2.3.4 Storage of Bacterial Stocks**

*E. coli* bacterial stocks (NEB® 5-alpha Competent *E. coli* (C2987H) or NEB® Turbo Competent *E. coli* (C2984H)) were stored at -80 °C in a 15% v/v glycerol solution.

## 2.4 MOLECULAR TECHNIQUES

### 2.4.1 Nucleic Acid Extraction and Preparation Techniques

#### 2.4.1.1 Small Scale Plasmid Preparation from Bacterial Colonies

Small scale plasmid extraction from bacterial colonies was performed using the NEB Monarch® Plasmid Miniprep Kit (NEB, T1010L). Up to 5 mL of bacterial culture was grown according to Section 2.4.1.1. From here, the NEB Monarch® Plasmid Miniprep Kit was performed according to manufacturer's instructions, eluting with 30 – 40 µL elution buffer.

Eluted DNA was then quantified using a nanodrop and stored according to Section 2.4.3.

#### 2.4.1.2 Large Scale Plasmid Preparation from Bacterial Colonies

For the large-scale extraction of plasmid from bacterial colonies, the Thermo Scientific™ GeneJET Plasmid Maxiprep Kit (K0492) was used. Up to 250 mL of bacterial culture was grown as described in Section 2.4.3 before the GeneJET Plasmid Maxiprep Kit was used according to the manufacturer's instructions. A total of one mL of elution buffer was used to elute DNA. Eluted DNA was then quantified using a nanodrop and stored according to Section 2.4.3.

#### 2.4.1.3 Extraction of RNA from Virus Culture Supernatant

RNA extraction from virus culture supernatant was performed using TRIzol™ LS Reagent (Invitrogen, 10296028). RNA extraction was performed according to the manufacturer's instructions, with 50 µL of RNase-free water being used to resuspend RNA. RNA was stored according to Section 2.4.3.

#### 2.4.1.4 Extraction of DNA from Agarose Gel Slices

The extraction of DNA from agarose gel slices was performed using the NEB Monarch® DNA Gel Extraction Kit (T1020L). Here, the Monarch® DNA Gel Extraction Kit was used according to the manufacturer's instructions, with 20 microliter (µL) of elution buffer being used to elute DNA from the purification column. Eluted DNA was then quantified using a nanodrop and stored according to Section 2.4.3.

#### 2.4.1.5 Extraction of DNA from PCR mix

To purify DNA from post-PCR mixes, the NEB Monarch® PCR & DNA Cleanup Kit (T1030L) was used. Here, the NEB Monarch® PCR & DNA Cleanup Kit was used according to the manufacturer's instructions, with 20 µL of elution buffer being used to elute DNA from the purification column. Eluted DNA was then quantified using a nanodrop and stored as described in Section 2.4.3

## 2.4.2 Polymerase Chain Reaction (PCR)

### 2.4.2.1 PCR Using Q5® DNA Polymerase

PCR was performed using NEB Q5® DNA Polymerase (M0492L). PCR mixes were made in the ratios given in Table 2:2.

| Component                              | Final Concentration    |
|----------------------------------------|------------------------|
| <b>Q5® High-Fidelity 2X Master Mix</b> | 1x                     |
| <b>10 µM Forward Primer</b>            | 0.5 µM                 |
| <b>10 µM Reverse Primer</b>            | 0.5 µM                 |
| <b>Template DNA</b>                    | 1 – 20 ng              |
| <b>Nuclease-Free Water</b>             | Top up to final volume |

Table 2:2: Components and final concentrations used in Q5® DNA polymerase reactions.

PCR was performed according to the manufacturer's instructions. In short, nuclease-free water was combined with both forward and reverse primers on ice. Template DNA was then added, followed by the Q5® High-Fidelity 2x Master Mix. PCR was performed according to Table 2:3, where the melting temperature ( $T_m$ ) for oligonucleotide primers had been determined by the NEB  $T_m$  Calculator (Section 2.27.2) or the built-in  $T_m$  calculators within the NEBaseChanger® (Section 2.27.3) or NEBuilder® (Section 2.27.4).

| Step                        | Temperature       | Time       |
|-----------------------------|-------------------|------------|
| <b>Initial Denaturation</b> | 98 °C             | 30 sec     |
| <b>25 – 35 Cycles</b>       | 98 °C             | 10 sec     |
|                             | Primer pair $T_m$ | 30 sec     |
|                             | 72 °C             | 30 sec /kb |
| <b>Final Extension</b>      | 72 °C             | 2 min      |
| <b>Hold</b>                 | 4 °C              |            |

Table 2:3: Incubation times and temperatures used when performing PCR using the Q5® High-Fidelity 2X Master Mix.

### 2.4.2.2 PCR for Bacterial Colony Screening

To screen for positive bacterial colonies (colony PCR) on Luria Bertani (LB) Agar plates, GoTaq® Green Master Mix (M7122) (Promega) was used. GoTaq® Green Master Mix was selected for this purpose due to the presence of two dyes which allowed immediate loading into electrophoresis agarose gels. PCR mixes per colony were made in a 15 µL volume in the ratios given in Table 2:4.

| Component                   | Final Concentration    |
|-----------------------------|------------------------|
| GoTaq® Green Master Mix, 2x | 1x                     |
| 10 µM Forward Primer        | 0.5 µM                 |
| 10 µM Reverse Primer        | 0.5 µM                 |
| Template DNA                | 1 – 20 ng              |
| Nuclease-Free Water         | Top up to final volume |

Table 2:4: Components and final concentrations used in GoTaq® Green PCR reactions used in screening for positive bacterial colonies.

Up to 20 colonies were screened simultaneously in colony PCR. To perform this, a PCR master mix was created to screen all desired colonies, including a 5% volume buffer to allow for pipetting error (i.e. 315 µL of master mix was created for 20 colonies). After aliquoting into separate 200 µL PCR tubes on ice, a pipette tip was used to take the edge of a bacterial colony before being briefly submerged in PCR mix. PCR was then performed according to Table 2:5, where the T<sub>m</sub> of oligonucleotide primer pairs was determined using the Promega T<sub>m</sub> for Oligos Calculator (Section 2.27.6).

| Step                 | Temperature                | Time      |
|----------------------|----------------------------|-----------|
| Initial Denaturation | 95 °C                      | 2 min     |
| 25 – 35 Cycles       | 95 °C                      | 1 min     |
|                      | Primer pair T <sub>m</sub> | 1 min     |
|                      | 72 °C                      | 1 min /kb |
|                      | 72 °C                      | 2 min     |
| Final Extension      | 72 °C                      | 2 min     |
| Hold                 | 4 °C                       |           |

Table 2:5: Details of PCR timings and temperatures used in colony PCR using GoTaq® Green Master Mix.

#### 2.4.2.3 Reverse Transcriptase PCR

To create complementary DNA (cDNA) from viral RNA extraction (Section 2.4.1.3) the QIAGEN OneStep RT-PCR Kit was used (Qiagen, 210212) according to the manufacturer's instructions. PCR primer T<sub>m</sub> were calculated using Promega T<sub>m</sub> for Oligos Calculator (Section 2.27.6) and performed according to Table 2:6 and Table 2:7.

| Step                   | Temperature                | Time   |
|------------------------|----------------------------|--------|
| Reverse Transcription  | 50 °C                      | 30 min |
| Initial PCR Activation | 95 °C                      | 15 min |
| 40 Cycles              | 94 °C                      | 1 min  |
|                        | Primer pair T <sub>m</sub> | 1 min  |
|                        | 72 °C                      | 1 min  |
|                        | 72 °C                      | 10 min |
| Final Extension        | 72 °C                      | 10 min |
| Hold                   | 4 °C                       |        |

Table 2:6: Incubation times and temperatures used when performing reverse transcriptase PCR on viral RNA to create complementary DNA.

| Component                        | Final Concentration or Volume (50 $\mu$ L Final Volume) |
|----------------------------------|---------------------------------------------------------|
| QIAGEN OneStep RT-PCR Buffer, 5x | 1x                                                      |
| dNTP mix (10 nM each)            | 400 $\mu$ M of each dNTP                                |
| 10 $\mu$ M Forward Primer        | 0.6 $\mu$ M                                             |
| 10 $\mu$ M Reverse Primer        | 0.6 $\mu$ M                                             |
| QIAGEN OneStep RT-PCR Enzyme Mix | 2 $\mu$ L                                               |
| RNA                              | 1 $\mu$ g                                               |
| RNase-Free Water                 | Top up to 50 $\mu$ L                                    |

Table 2:7: Components and final concentrations used in QIAGEN OneStep RT-PCR reactions.

Final volume of all reactions was 50  $\mu$ L

#### 2.4.3 Storage of Nucleic Acids

All DNA extracted from bacterial cultures (Sections 2.4.1.1 and 2.4.1.2), agarose gel slices (Section 2.4.1.4), and PCR mixes (Section 2.4.1.5), was stored at -20 °C in the elution buffers provided in their respective extraction and purification kits. Any un-extracted PCR reaction mixes or agarose gel slices were stored at -20 °C or 4 °C, respectively.

All RNA extracted from virus culture supernatant (Section 2.4.1.3) was stored in RNase-free water at -80 °C.

#### 2.4.4 Restriction Enzyme Digestion

Restriction digestion of DNA was used throughout this project primarily to remove any contaminating DNA from PCR reactions. In cases where the concentration of DNA was known, up to one  $\mu$ g of DNA was digested at once in a final volume of 25  $\mu$ L. In cases where the concentration of DNA was unknown (such as in PCR for HiFi Assembly (Section 2.8)) one  $\mu$ L of enzyme per 50  $\mu$ L of total volume was used. All restriction enzymes were incubated and inactivated (where possible) according to the manufacturer's (New England Biolabs) instructions.

#### 2.4.5 Agarose Gel Electrophoresis

Agarose powder (Bioline, BIO-41025) was used to make 1% w/v agarose gels in 1x TAE buffer (Section 2.1). After the addition of agarose powder to TAE, the mixture was briefly microwaved for two minutes 30 seconds in a cat 900 microwave. After cooling, a 1/1000 dilution of SYBR<sup>TM</sup> Safe DNA Gel Stain (Invitrogen, S33102) was added and the gel left to set at room temperature for 30 min.

Up to one  $\mu$ g of sample was mixed with Blue/Orange Loading Dye, 6X (Promega, G1881) for efficient loading and visualisation before being run on agarose gels. A DNA ladder was also included on each agarose gel, using the Promega 1kb DNA Ladder (G5711) or NEB Quick-Load<sup>®</sup> 1 kb Extend DNA Ladder.

#### 2.4.6 SDS-PAGE electrophoresis and Western Blot

Before samples were separated on a sodium dodecyl sulphate (SDS) - polyacrylamide gel electrophoresis (PAGE) gel, samples were incubated at 95 °C with 4x NuPAGE™ LDS Sample Buffer (Invitrogen, NP0007) for five min. Samples were then ran on a pre-made NuPAGE 4-12% Bis-Tris Gels (Invitrogen, NP0322BOX) with MOPS running buffer (Section 2.1). SDS-PAGE gels were then transferred to a nitrocellulose blotting membrane (Merck, GE10600003) before being blocked by a milk-TBS blocking solution (Section 2.1) overnight.

For visualisation of mCHERRY, an anti-red fluorescent protein (RFP) antibody (6G6, Chromotek) was incubated on previously blocked nitrocellulose membranes at a 1/1000 dilution in a milk-TBS blocking solution (Section 2.1). After three five-minute washes with TBS-Tween 20, anti-RFP antibody was bound by a secondary anti-Mouse horseradish peroxidase (HRP) antibody (Merck, A2554) at a 1/1000 dilution in milk-TBS blocking solution (Section 2.1) for one hour at room temperature. HRP was visualised using the ECL™ Prime Western Blotting System (Merck, GERPN2232).

## 2.5 SEQUENCING

### 2.5.1 Sanger Sequencing

PCR product or plasmid DNA were sequenced by the Central Sequencing Unit (CSU) at the Animal and Plant Health Agency. Samples were cleaned and dye terminated products produced using the BigDye Terminator v3.1 Cycle sequencing kit (Applied Biosystems). These products were then run on ABI 3130xl (16 capillary) and ABI 3730 (48 capillary) Genetic Analysers to obtain sequence reads.

### 2.5.2 Next Generation Sequencing

Next generation sequencing was performed by the CSU at the Animal and Plant Health Agency. DNA was first purified using AMPure XP magnetic beads (Beckman Coulter) and 1 nanogram (ng) was used in the Nextera XT DNA sample preparation kit (Illumina) to fragment and tag DNA. After a library was prepared according to the manufacturer's instructions this was sequenced Illumina MiSeq with two 150 base pair (bp) paired-end reads according to the manufacturer's instructions. The resulting reads were mapped to a reference sequence using the Burrows-Wheeler Aligner (BWA) (version 0.7.17) and were visualised in Tablet <sup>315</sup>.

A modified SAMtools/vcfutils script <sup>316</sup> was used to generate an intermediate consensus in which any indels relative to the original reference sequence were appropriately called. The intermediate consensus sequence was used as the reference for subsequent iteration of mapping and consensus calling

## 2.6 BACTERIAL TRANSFORMATION AND CULTURE

### 2.6.1 Bacterial Transformation

Bacterial transformation by heat-shock was performed the same for both NEB® 5-alpha Competent *E. coli* (C2987H) and NEB® Turbo Competent *E. coli* (C2984H). First, >10 ng (unless specified otherwise in sections 2.8 or 2.9) of DNA was mixed with 50 µL of *E. coli* after defrosting on ice. This was incubated for one to four h on ice before briefly submerging in a 42 °C water bath for 30 sec. After the addition of 450 µL of super optimal broth with catabolite repression (SOC) media, bacterial solutions were incubated for one h at 37 °C in a shaking incubator (220 rpm). From here, bacterial colonies were cultured according to Section 2.6.2.

### 2.6.2 Bacterial Culture on Agar Plates

Antibiotic-containing agar plates were made by melting solid LB agar in a microwave and leaving to cool. After cooling, either ampicillin or kanamycin antibiotic was added to give a final concentration of 100 or 50 µg /mL, respectively. After thorough mixing, agar was left to set in 10 cm round dishes at room temperature.

If culturing after bacterial transformation (Section 2.6.1), either 40 µL of suspension was pipetted onto the agar plate and spread using a spreader, or the suspension was centrifuged to pellet bacteria and resuspended in 40 µL of SOC media. The entire 40 µL volume was then pipetted onto the agar plate and spread using a bacterial spreader.

If culturing from a -80 °C glycerol stock, a pipette tip was used to collect frozen bacteria, which was then gently spread onto an agar plate to allow effective isolation of a single colony. Bacterial plates were then incubated upside down for 16 h or overnight at 37 °C 5% CO<sub>2</sub>.

### 2.6.3 Bacterial Culture in LB Broth

Single bacterial colonies were taken and grown in LB agar broth. Either five or 250 mL of LB broth was grown for plasmid extraction by miniprep (Section 2.4.1.1) and maxiprep (Section 2.4.1.2) respectively. In all applications, an antibiotic for selection pressure was included. Either ampicillin or kanamycin was added to give a final concentration of 100 or 50 µg /mL. Cultures were then incubated for 16 h or overnight at 37 °C in a shaking incubator (220 rpm).

## 2.7 NANO-GLO® LUCIFERASE ASSAY

Luciferase assays using the Nano-Glo® Luciferase Assay System (NEB, N1130) were performed according to the manufacturer's instructions. All reagents were stored at -20 °C. Before use, one aliquot of Nano-Glo® Luciferase Assay Substrate (200 µL) and Nano-Glo® Luciferase Assay

Buffer (10  $\mu$ L) were thawed and mixed thoroughly. This solution was protected from light whenever possible. After use, any remaining solution was stored at -20 °C.

To eliminate any inter-well interference from luminescence, all cell culture was performed in 96-well clear-bottomed opaque (white)-sided tissue culture plates (Corning, 3610). To analyse luciferase expression both within mammalian cells and in the supporting supernatant, the 'lytic method' was used. Here, all assay components were equilibrated to room temperature (both the tissue culture plate and assay reagents). Up to 50  $\mu$ L of tissue culture supernatant was removed to leave a total of 50  $\mu$ L remaining. 50  $\mu$ L of Nano-Glo<sup>®</sup> Luciferase Assay Reagent was then added to each well, mixed gently, and incubated in the dark for 10 minutes. Luminescence was then read using a GloMax<sup>®</sup>-Multi Detection System (Promega). Luminescence values are displayed in Relative Luminescence Units (RLU) as a fold increase from the negative control (cell only).

To analyse luciferase expression in supernatant only, 50  $\mu$ L of supernatant was removed from each tissue culture well and pipetted into a clear-bottomed opaque-sided tissue culture plate (Corning, 3610). The luciferase assay was then performed as described above.

## 2.8 NEBUILDER<sup>®</sup> HiFi DNA ASSEMBLY

All DNA cloning using NEBuilder<sup>®</sup> HiFi DNA Assembly was performed using the NEBuilder<sup>®</sup> HiFi DNA Assembly Cloning Kit (E5520S, NEB). This was performed according to the manufacturer's instructions with some alternations described below.

To generate fragments for HiFi DNA assembly, 100  $\mu$ L PCRs were performed according to Section 2.4.2.1 using the oligonucleotide primer/template combinations described in Section 2.21. Contaminating template DNA was removed from PCRs according to Section 2.4.4 using enzyme *DpnI*. The desired PCR products were purified from solution according to Section 2.4.1.5 and a gel electrophoresis performed to confirm correct PCR band size (Section 2.4.5).

To assemble fragments, 0.05 picomole (pmol) of each DNA PCR fragment was combined in a 200  $\mu$ L PCR tube and mixed with an equal volume of NEBuilder HiFi DNA Assembly Master Mix. Depending on how many fragments were being simultaneously assembled the mixture was incubated for either 15 min (two/three fragments) or 60 min (four-six fragments) at 50 °C. After incubation, assembly reactions were digested a second time with *DpnI* (Section 2.4.4).

As specified by the manufacturer, two  $\mu$ L of assembly reaction was used to transform competent bacterial cells as according to Section 2.6.1. Bacterial colonies were then screened for positive colonies using Colony PCR (Section 2.4.2.2) and cultured in LB broth containing an appropriate antibiotic for plasmid extraction and downstream applications.

Amounts of DNA fragments used in HiFi assembly was calculated using “Bens\_Gibson\_Assembly” Gibson calculator (Barrick Lab, University of Texas, Austin, <https://barricklab.org/twiki/bin/view/Lab/ProtocolsGibsonCloning>).

## 2.9 Q5® SITE-DIRECTED MUTAGENESIS

Site directed mutagenesis was performed using the Q5® Site-Directed Mutagenesis Kit (E0554S, NEB) according to the manufacturer’s instructions.

Briefly, a PCR mix was created using primers designed using the online NEBaseChanger® software (Section 2.27.3) and ran for 25 cycles using the temperature specified. After PCR cycling, a KLD reaction mix was made as shown in Table 2:8. This mixture was incubated for an extended 30 minutes at room temperature. Five µL was then transformed into competent bacterial cells as according to Section 2.6.1. Bacterial colonies were then screened for positive colonies using Colony PCR (Section 2.4.2.2) and cultured in LB broth containing an appropriate antibiotic for plasmid extraction and downstream applications.

| Component              | Final Volume or Concentration |
|------------------------|-------------------------------|
| 1x PCR Mix             | 1 µL                          |
| 2x KLD Reaction Buffer | 6 µL                          |
| 10x KLD Enzyme Mix     | 1 µL                          |
| Nuclease-Free Water    | 2 µL                          |

Table 2:8: Components and volumes used in the NEB KLD reaction mix

## 2.10 FUGENE TRANSFECTION OF DNA INTO CELL CULTURE

Upon reaching 70-80% mammalian cell monolayer confluence within a 96-well plate (Corning), cell culture media was replaced with 90 µL of fresh, pre-warmed, media (Table 2:1). DNA transfection into cell culture was performed using FuGENE® 6 Transfection Reagent (Promega, E2692) according to the manufacturer’s instructions (10 µL final volume per 96-well was used). Briefly, FuGENE® 6 Transfection Reagent was mixed with OptiMEM and incubated at room temperature for five min. FuGENE® 6 Transfection Reagent was used at a 3.5:1 DNA ratio. After incubation, DNA (if multiple different plasmids were used these were combined before addition to FuGENE – OptiMEM mix) was added to the FuGENE – OptiMEM mix and incubated for a further 30 min at room temperature. After incubation, FuGENE-OptiMEM-DNA mix was added dropwise to wells. The cell culture plate was gently agitated and incubated at 37 °C 5% CO<sub>2</sub>.

## 2.11 INFECTION OF CELL CULTURE WITH FOWLPOX-T7 VIRUS

To assess T7 expression after Fowlpox-T7 infection,  $3 \times 10^4$  BHK-21 cells per well were seeded onto a single 96-well plate (Corning) and incubated at 37 °C 5% CO<sub>2</sub>. After 16 h, three wells were trypsinised and counted. Fowlpox-T7 virus was then defrosted from -80 °C and diluted in cell culture media (Table 2:1) to reach a multiplicity of infection (MOI) of one, two, three, four, or five for BHK-21 infection. To infect cells, media from wells was removed and Fowlpox-T7 incubated on cells for one h at 37 °C, cells were then washed with PBS and cell culture media added.

## 2.12 RABIES VIRUS RESCUE AND CULTURE

### 2.12.1 Rabies Virus Rescue from cDNA

Rabies virus rescue involves the simultaneous transfection of three RABV helper plasmids (pTIT-N, P, L) alongside a full-length RABV plasmid in the presence of T7 polymerase <sup>79</sup>. In classical RABV rescue, the mammalian cell-line BHK-21 is used, and a T7-encoding plasmid is transfected alongside the aforementioned helper/full-length RABV plasmids. However, in this study the cell line BSR-T7/5 was used. BSR-T7/5 cells are a derivative of BHK-21 cells which have been stably transfected with a T7 RNA polymerase gene, eliminating the need for transfection of a T7 polymerase-expressing plasmid <sup>317</sup>.

18 h before transfection,  $3 \times 10^4$  BSR-T7/5 cells were seeded per well in a 96-well plate (Corning) in 100 µL of Glasgow Minimum Essential Medium (GMEM) (Gibco) media supplemented with 5% FBS and pen-strep (10 U /mL penicillin, 10 µg/mL streptomycin) (Table 2:1). 40 min before transfection, all DNA was removed from -20 °C storage and defrosted on ice. Per well, 40 ng of RABV-helper plasmids (pTIT-N, pTIT-P, pTIT-L <sup>318</sup>) and 200 ng of full-length RABV construct was simultaneously transfected using FuGENE® 6 Transfection Reagent (Promega) at a 3.5:1 ratio in 10 µL of total transfection mix. The 96-well plate was then gently agitated before incubation for four days at 37 °C 5% CO<sub>2</sub>. After four days, cells were passaged into 12-well plates (Corning) at a ratio of three 96-wells to a single 12-well. Cells were incubated for four days (37 °C 5% CO<sub>2</sub>) or until confluency. This pattern was repeated, passaging to larger plates/flasks, until a suitable volume of media (approximately 80 mL) was achieved. To harvest virus, supernatant was aspirated and cells freeze-thawed three times. Supernatant and cell lysate was combined and centrifuged at 5300 x g for five minutes before being stored at -80 °C.

Successful virus rescue was determined through the observation of enlarging fluorescent foci over multiple passages.

### 2.12.2 Virus Titration

The titre of cultured viruses was determined in focus-forming units per mL (FFU/mL). Each virus titration was performed across a 96-well plate in eight serial 10-fold dilutions. In short, eight tubes were aliquoted with 1800  $\mu$ L GMEM cell culture media. The first tube was mixed with 200  $\mu$ L virus, resuspended, and 200  $\mu$ L taken from this tube into the next. After eight serial dilutions were performed, 100  $\mu$ L was taken from each tube and aliquoted into the 96-well plate.  $5 \times 10^5$  BHK-21 cells were then added to each titration well and incubated for 48 h at 37 °C 5% CO<sub>2</sub>.

To image virus infection in cell culture, acetone was used to fix cells (Section 2.16) before immunocytochemistry performed on the RABV N protein. Briefly, after drying wells following acetone fixation, 50  $\mu$ L of a 1:100 dilution of anti-N FITC conjugated mAb (Fujirebio) was added to each well and incubated for 30 min at 37 °C. Plates were then washed three times in 0.1 molar (M) PBS (Section 2.1) and thoroughly dried. Immunofluorescence was read under a UV microscope, where foci of cell specific apple green perinuclear fluorescence indicated the presence of RABV N protein. The number of green foci in each well was recorded and the number of foci in the last well before no foci was present was used to calculate FFU/mL.

### 2.13 SUBCULTURE OF VIRAL STOCKS TO HIGH TITRES

In experiments which required high titres of virus. Viruses were passaged to achieve a higher titre than the originally generated stock. Here,  $3 \times 10^5$  BHK-21 cells were cultured per well in a 12-well plate. After 24 h incubation at 37 °C, media was removed cell culture media containing virus was added to a final volume of one mL per well. Wells were then cultured for 72 h before virus-containing supernatant aspirated, mixed in a 1/2 dilution with fresh media, and added on to pre-plated cells as described previously. This process was performed three times in total before media was aliquoted for subsequent storage and titration (Section 2.12.2).

### 2.14 MULTI-STEP VIRUS GROWTH CURVES

All multi-step growth curve experiments were performed in 12-well plates (Corning) using BHK-21 cells. Each growth curve was repeated in three separate wells and samples taken at timepoints 0, 6, 12, 24, 48, 72, 96, 120 h. First,  $1.5 \times 10^5$  BHK-21 cells were added per well and supplemented with a total of two mL of GMEM (Table 2:1). After incubation overnight, three wells were trypsinised with one mL of ATV each (Section 2.3.1) and counted.

All wells were then infected at an MOI of 0.01 in a final well volume of one mL. After one h incubation at 37 °C, all media was removed and washed three times with PBS (Section 2.1) before being replaced with two mL of room-temperature GMEM. At timepoints 0, 6, 12, 24,

48, 72, 96, and 120 h, 200 µL of media was removed from each well and replaced with an equal volume of fresh media. Removed media was then replaced with fresh GMEM. All timepoint aliquots were immediately stored at -80 °C.

After the last timepoint was taken and removed media frozen at -80 °C, all timepoint aliquots were thawed to room temperature and analysed by virus titration as described in Section 2.12.2.

## 2.15 ENZYME-LINKED IMMUNOSORBENT ASSAY

In this thesis, the enzyme-linked immunosorbent assay (ELISA) protocol used was based upon the Platelia® Rabies II Kit (BioRad) with major experimental modifications. Samples were diluted in the Platelia® Rabies II Kit (BioRad) R6 reagent [Tris – Ethylenediaminetetraacetic acid (EDTA) buffer] and 100 µL incubated for one h at 37 °C on the pre-coated Platelia® RABV G (Pitman-Moore strain) protein ELISA wells. After washing wells three times with PBS, 100 µL of a 1/1000 dilution of a rat anti-mCHERRY (5F8, chromotek) antibody was incubated for one h at 37 °C. After a further three washes, wells were incubated for one h at 37 °C with an anti-rat HRP-labelled antibody (405405, BioLegend). After three washes with PBS, 100 µL of Platelia® Tetramethylbenzidine (TMB) solution [a 1/11 dilution of solutions R9 (TMB solution, 0.25%) and R8 (citric acid and sodium acetate containing 0.015% H<sub>2</sub>O<sub>2</sub> and 4% dimethylsulfoxide)] was added to each well and Platelia® solution R9 (0.5 M sulphuric acid solution) used to stop the reaction.

In total, three experiments were performed to investigate the production of 62-71-3 scFv over time in RABV-62scFv infected cell culture. First, a single-step growth curve was performed in BHK-21 cells with both RABV-62scFv and RABV-mCH-K226R. A single-step growth curve (as opposed to a multi-step growth curve) is identical to a multi-step growth curve, however, cells are instead infected with an excess of virus (five MOI). After samples were taken at 0, 4, 8, 24, 72, and 96 h post-infection they were stored at -80 °C prior to use. The second experiment was used to establish the appropriate dilution for samples for ELISA-based analysis. This involved serial dilution of a 96 h timepoint sample (selected as samples from this timepoint were suspected to have the highest concentration of scFv) and analysis by ELISA as described above. From this experiment, a 1/20 dilution was selected (data not shown). The final experiment involved the 1/20 dilution of all RABV-62scFv and RABV-mCH-K226R samples and analysis by ELISA. All OD450 values were expressed as a fold change from the negative media-only control.

## 2.16 CELL CULTURE FIXATION AND RABV N IMMUNOCYTOCHEMISTRY

To fix cells infected with virus in acetone, all culture supernatant was removed from tissue culture plate wells and replaced with fresh 80% v/v acetone solution in distilled water. This was then discarded and replaced with 80% v/v acetone solution in distilled water for 20 min. Plates were then washed twice in PBS (Section 2.1) and Fujirebio FITC Anti-Rabies Monoclonal Globulin (800-092) (1/100 dilution) added to each well and incubated at 37 °C for 30 minutes. Following incubation, anti-Rabies antibody was removed, and wells washed three times with 0.1 M PBS (Section 2.1). After drying, wells were visualised on a UV microscope for presence of RABV N protein.

## 2.17 FLUORESCENT ANTIBODY VIRUS NEUTRALISATION TEST

To perform a fluorescent antibody virus neutralisation (FAVN) test, 100 µl of GMEM cell-culture media was first added to rows E-H of a 96-well plate (Corning). 100 µl of GMEM was then added to wells A1-D1, and 150 µl GMEM to A2-D6. 50 µl of virus-produced 62-71-3 (62-71-3) supernatant was then added to wells E1-H1 and serially diluted across the plate. 50 µl of challenge virus at 100 TCID<sub>50</sub> was subsequently added to wells E1-H12. Alongside this, a virus titration was prepared to confirm the correct dilution of the aforementioned challenge virus. 50 µl of challenge virus at 100 TCID<sub>50</sub> was added to wells A1-D2 and serially diluted from A2-D2 to A6-D6. After a one h incubation at 37 °C 5% CO<sub>2</sub>, 50 µl of BHK-21 cells were added to all wells for a final concentration of 3x10<sup>4</sup> cells /well. FAVN plates were then incubated for 48 h at 37 °C 5% CO<sub>2</sub>. FAVNs were performed in triplicate.

After acetone fixation and RABV N protein visualisation (Section 2.16), the 50% endpoint dilution (where complete neutralisation appeared in 50% of wells) was calculated using the Spearman-Kärber method <sup>319</sup>. Antibody titre in IU /mL was calculated by comparing the 50% endpoint dilution to a standard control at 0.5 IU /mL.

## 2.18 FLUORESCENT ANTIBODY TEST

To visualise RABV N protein within the brains of mice, a small amount of brain tissue was removed from the hindbrain and placed on blotting paper. In this test, brain from a known-positive animal was included as a positive control. Using the blotting paper, two impressions were made on a glass microscope slide and any excess removed by extra blotting. After air drying to two to three minutes, slides were fixed in 100% acetone for 20 min at rt. After removal from acetone, anti-RABV N protein FITC antibody (Fujirebio) was added to the sample and incubated for a further 30 min at 37 °C. After two washes in PBS, and a single wash in H<sub>2</sub>O, slides were dried and fluorescence observed by illumination under UV.

## 2.19 RABIES TISSUE CULTURE ISOLATION TEST

The presence of live virus in mouse brain was assessed using the rabies tissue culture isolation test (RTCIT). Here, a 10% w/v homogenate was made of brain in PBS. 100 µL of brain homogenate was then added to N2a cells which had been plated out 30 min beforehand [4x10<sup>4</sup> cells per well in 200 µL of GMEM (Section 2.3.1)]. Similar to Section 2.18, brain from a confirmed positive mouse was used as a positive control. N2a cells were incubated for 24 h at 37 °C before the media was replaced and incubated for a further four days. Here, media was removed, cells fixed in acetone (Section 2.16), stained with an anti-RABV N protein FITC antibody (Fujirebio) and observed for the presence of apple-green perinuclear fluorescence.

## 2.20 MTS CELL VIABILITY ASSAY

Assessment of the toxicity of virus infection was performed using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) (Promega) according to the manufacturer's instructions. N2a (ATCC® CCL-131™) cells were cultured in a 96-well plate (Corning) at a density of 4x10<sup>4</sup> cells /well. 24 h after seeding, N2a cells were infected with virus at a MOI of 0.01. N2a cells were then incubated for 48 h at 37 °C 5% CO<sub>2</sub>. To assess cell viability, room-temperature CellTiter 96® AQueous One Solution Reagent was added to each well at a 1/6 dilution and incubated for 4 h at 37 °C 5% CO<sub>2</sub>. Absorbance was later read at 450 nm using a Promega GloMax®-Multi Detection System Plate Reader.

Analysis of results was performed using the following formula and displayed as a percentage reduction of cell viability compared to uninfected cells.

$$\frac{\text{Absorbance Value of Infected Cell}}{\text{Absorbance Value of Uninfected Cell Control}} \times 100$$

## 2.21 ASSESSING KINETICS OF CELL-TO-CELL SPREAD OF RABIES VIRUSES

The ability of RABVs to spread between cells was assessed by infecting a monolayer of either N2a or BHK-21 cells with a low concentration of virus particles and observing the number of cells per foci at various time points. Briefly, 3x10<sup>6</sup> BHK-21 cells per-well were seeded in a 6-well plate (or 1x10<sup>7</sup> cells seeded in a 10 cm dish) before incubation overnight at 34 °C 5% CO<sub>2</sub>. Confluent cell monolayers were then infected with 0.001 MOI of virus for one hour at 37 °C 5% CO<sub>2</sub>. Cell monolayer was then washed three times in PBS 1.5 mL before a 0.3% agarose-GMEM overlay (Section 2.1) mix was gently added. Agarose-GMEM was then allowed to set at room temperature for 10 min before being incubated at 37 °C 5% CO<sub>2</sub>. At 24, 48, 72, 96, and 120 h post-infection, cells were fixed in 80% acetone (Section 2.16) for 20 min before a 1:100 dilution of anti-N FITC conjugated mAb (Fujirebio) was added to each well and incubated for 30 min at

37 °C. Plates were then washed three times in 0.1 M PBS and thoroughly dried. 100 foci were identified under UV microscope by apple green perinuclear fluorescence.

## 2.22 USING A hCMEC/D3 TRANSWELL ASSAY TO ASSESS THE ABILITY OF RABV-62scFv TO CROSS THE BBB

### 2.22.1 Formation of the hCMEC/D3 BBB model

To culture hCMEC/D3 cells on semi-permeable tissue culture membrane transwell inserts (3.0  $\mu\text{m}$  pore size <sup>320</sup>, Corning, 353181), these were placed in a 12-well plate (Corning) and 400  $\mu\text{L}$  (0.15 mg/mL) of rat tail collagen (Merck, 08-115) incubated on inserts at 37 °C, 5% CO<sub>2</sub>. After one h, collagen was then removed, and the insert washed with PBS.  $5 \times 10^4$  hCMEC/D3 cells were then seeded on each insert and incubated at 37 °C, 5% CO<sub>2</sub>. Once seeded on semi-permeable inserts, media was replaced every other day.

### 2.22.2 Assessing permeability of the hCMEC/D3 BBB model using Lucifer Yellow

Permeability of hCMEC/D3 monolayers was assessed using Lucifer yellow (LY) (Merck) at 24 h intervals after seeding on cell culture inserts as described by Zhao et al <sup>321</sup>. After removal of growth media, hCMEC/D3 monolayers (and surrounding well) were washed with pre-warmed PBS. After removal of PBS, 175  $\mu\text{L}$  of 20  $\mu\text{M}$  LY (diluted in transport buffer, Section 2.1) was added to the apical side of the cell culture insert and 1.5 mL of transport buffer added to the basolateral side. These wells were then incubated at 37 °C 5% CO<sub>2</sub> on a microplate shaker (100 rpm). After one h incubation, samples were taken from both the apical (diluted 1/10 in transport media) and basolateral side. These, alongside seven LY standards (2500, 1250, 625, 312.5, 156.25, 78.125, 39.0625 nM) and the original 20  $\mu\text{M}$  stock of LY added to the apical side (diluted 1/10 in transport media), were added to a black opaque-sided plate (Corning, 3904) and read on a fluorescent plate reader at excitation 428 nm, emission 536 nm.

The values obtained from the standards were used to generate a standard curve in GraphPad. A simple linear regression line was fitted to these values and used to interpolate LY concentrations for all samples taken. To determine the permeability of the hCMEC/D3 monolayer, a  $P_{\text{app}}$  (permeability of the hCMEC/D3 barrier) and LY recovery % (how much LY was lost during the experiment) value was generated. The method used to generate these values is described below.

To calculate the LY recovery percentage, the following formula was used. Where  $M_{\text{Af}}$  was the concentration ( $\mu\text{M}$ ) of LY in the sample taken from the apical side, and  $M_{\text{Bf}}$  the concentration

( $\mu\text{M}$ ) of LY in the sample taken from the basolateral side.  $M_{A0}$  was the initial concentration of LY added to the apical side prior to incubation.

$$\text{Recovery \%} = \frac{M_{Af} + M_{Bf}}{M_{A0}} \times 100$$

To calculate  $P_{app}$ , the following formula was used. Here,  $V_B$  was the volume added to the basolateral compartment,  $A$  was the surface area of the cell culture insert,  $C_{A0}$  was the initial concentration of LY added to the apical compartment, and  $\Delta CB/\Delta t$  was the change of LY concentration over time within the basolateral compartment.

$$P_{app} \left( \frac{\text{cm}}{\text{min}} \right) = \frac{V_B}{A \times C_{A0}} \times \left( \frac{\Delta CB}{\Delta t} \right)$$

This experiment was performed every 24 h for 10 days after hCMEC/D3 cells were seeded on the cell culture transwell inserts.

### 2.22.3 Assessing the ability of RABV-62scFv to cross a hCMEC/D3 BBB model

To assess the ability of RABV-62scFv to cross the hCMEC/D3 BBB model, after the hCMEC/D3 monolayer was allowed to form a barrier (determined by  $P_{app}$  values taken over 10 days) an infectious dose of RABV-62scFv (the highest concentration available) was added to the apical compartment and incubated on a shaking incubator 37 °C 5% CO<sub>2</sub> (100 rpm) for one h. Alongside this, an equivalent concentration of heat-inactivated RABV-62scFv was also activated on hCMEC/D3 cells. After incubation, RABV-62scFv (live or inactivated) was removed from cells, cells were washed three times with PBS and replaced with growth media. After 48 h, samples were taken from the basolateral side of the cell culture insert and RNA extracted (Section 2.4.1.3). These samples were then assessed for presence of viral RNA by RT-PCR as described in Section 2.4.2.3.

## 2.23 REVERSE GENETICS SCHEMATICS AND SEQUENCES OF OLIGONUCLEOTIDES

PCR, assembly of PCR fragments, and site-directed mutagenesis were performed as described in Sections 2.8 and 2.9, respectively. All products of cloning were confirmed by Sanger sequencing (Section 2.5.1).

Where ORFs were inserted in the RABV-cSN vector, to avoid the appearance of any mutations from extended PCR reactions the RABV-cSN vector was linearised into two ~8kb separate PCR products, instead of a single ~16kb PCR product.

#### 2.23.1 Generation of pTIT-NLuc construct to assess T7-polymerase expression.

Nanoluciferase (NLuc) was amplified from an NLuc-expressing vector, pNL1.1.CMV[*NLuc*/CMV] (Promega, N1091), using oligonucleotide primers 61 and 62. After linearising the pTIT-N plasmid (used in viral rescue) backbone using oligonucleotide primers 60 and 63, NLuc was used to replace the N gene by NEB NEBuilder® HiFi DNA Assembly (Section 2.8). Sequences of all primers used are listed in Appendix 2.

#### 2.23.2 Generation of pNL1.1 sec62-NLuc and pNL1.1 62-NLuc

Oligonucleotide primer pairs 55/56 or 59/56 were used to amplify the 62-71-3 scFv – 8xHis – E-tag cassette (not including DsRED). Oligonucleotide primers 55/56 included the secretory signal peptide, while 59/56 did not. The pNL1.1 vector was linearised using oligonucleotide primers 57 and 58. Using NEB NEBuilder® HiFi DNA Assembly (Section 2.8), 62-71-3 scFv fragments (generated by 55/56 or 59/56) were then inserted into the linearised pNL1.1 vector, so that NLuc was linked in-frame with 62-71-3 scFv similar to DsRed. This resulted in the creation of constructs termed pNL1.1 sec62-NLuc and pNL1.1 62-NLuc. Sequences of these primers are listed in Appendix 2.

#### 2.23.3 Insertion of the 62-71-3 scFv ORF within RABV-cSN

To insert the 62-71-3 scFv ORF before the N gene of the RABV-cSN plasmid, oligonucleotide primers 3 and 34 were used to amplify the 62-71-3 scFv – 8xHis – E-tag – DsRED ORF. To linearise the RABV-cSN vector, oligonucleotide primer pairs 1/30 and 4/31 were used to linearise the RABV-cSN vector in two parts. NEB NEBuilder® HiFi DNA Assembly (Section 2.8) was then used to assemble all PCR fragments. This construct was named RABV-62-H-E-DsR. Sequences of these primers are listed in Appendix 2.

#### 2.23.4 Replacement of DsRED of the 62-71-3 scFv ORF with mCHERRY

mCHERRY was amplified from an existing RABV-mCHERRY plasmid using oligonucleotide primers 120 and 70. RABV-62-H-E-DsR (Section 2.23.1) was linearised using oligonucleotide primer pairs 119/30 and 71/31. NEB NEBuilder® HiFi DNA Assembly (Section 2.8) was then performed to assemble all PCR fragments. This construct was named RABV-62-H-E-mCH. Sequences of these primers are listed in Appendix 2.

#### 2.23.5 Mutation of RABV-cSN G protein at residue 226

Oligonucleotide primers 64 and 65 were used to induce a K226R mutation (AAG to AGG) using the Q5® Site-Directed Mutagenesis Kit (Section 2.9) within the pTIT-G vector. RABV-K226R-G was then amplified using oligonucleotide primer pair 79/80. RABV-62-H-E-DsR was then linearised using oligonucleotide primer pairs 79/80 and 81/75. All fragments were then

assembled using NEB NEBuilder® HiFi DNA Assembly (Section 2.8) to form RABV-62-H-E-mK. Sequences of these primers are listed in Appendix 2.

#### 2.23.6 Alteration of the transcriptional start and stop units of the 62-71-3 scFv ORF

Oligonucleotide primers 133 and 134 were used to amplify the 62-71-3 scFv cassette within the RABV-62-H-E-mK construct. RABV-cSN was amplified in three parts to allow the insertion of RABV-K226R-G created in Section 2.23.5, using oligonucleotide primers pairs; 113 and 30; 135 and 78; and 81 and 31. RABV-K226R-G was amplified by oligonucleotide primers 79 and 80. NEB NEBuilder® HiFi DNA Assembly (Section 2.8) was then performed to assemble all fragments. This construct was named, in part due to the addition of a PolyA sequence, RABV-62-H-E-mK-PolyA. Sequences of these primers are listed in Appendix 2.

#### 2.23.7 Replacement of the RABV G gene in RABV-cSN with the IKOV G gene

To replace the G gene of RABV-62-H-E-mK-PolyA with the G gene from the *Ikoma Lyssavirus* (IKOV), IKOV G was first amplified using oligonucleotide primers cSN IKOV Fwd (forward) and cSN IKOV Rev (reverse), while oligonucleotide primer pairs 78/1841 and 81/1840 were used to linearise RABV-62-H-E-mK-PolyA. NEB NEBuilder® HiFi DNA Assembly (Section 2.8) was then used to assemble all fragments and the resulting construct named RABV-62-H-E-mCH-IKOV. Sequences of these primers are listed in Appendix 2.

#### 2.23.8 Replacement of 62-71-3 with murine TNF $\alpha$

In the replacement of the 62-71-3 scFv gene from RABV-62-H-E-mK-PolyA with the tumour necrosis factor  $\alpha$  (TNF $\alpha$ ) gene (Addgene:28089) <sup>322</sup>, two constructs were created. The first, named RABV-TNF $\alpha$ -H-E-mK, consisted of full-length TNF $\alpha$  substituted for the 62-71-3 scFv gene. The second construct, termed RABV-TNF $\alpha$ (mem)-H-E-mK, had the cleavage site abolished resulting in a non-secreted TNF $\alpha$ . The TNF $\alpha$  containing plasmid, GFP-TNF $\alpha$ , was a gift from Jennifer Stow (Addgene plasmid #28089) <sup>322</sup>.

To create RABV-TNF $\alpha$ -H-E-mK, oligonucleotide primers 146 and 157 were used to amplify TNF $\alpha$  while RABV-62-H-E-mK-PolyA (Section 2.23.6) was linearised using oligonucleotide primers 141 and 30, and 144 and 31. NEB NEBuilder® HiFi DNA Assembly (Section 2.8) was then performed to assemble all PCR fragments. To create RABV-TNF $\alpha$ (mem)-H-E-mK, TNF $\alpha$  underwent site-directed mutagenesis (Section 2.9) using oligonucleotide primers 158 and 159 to form TNF $\alpha$ (mem). TNF $\alpha$ (mem) was then amplified using oligonucleotide primers 146 and 157, before being inserted into RABV-62-H-E-Poly(A) as previously described. Sequences of these primers are listed in Appendix 2.

### 2.23.9 Removal of the 8xHis and E-Tag from the 62-17-3 scFv ORF within RABV-cSN

Two pairs of oligonucleotide primer pairs were used to amplify the two fragments of the 62-71-3 scFv ORF adjacent to the 8xHis and E-tag (62-71-3 scFv and mCHERRY, respectively). 62-71-3 scFv was amplified using oligonucleotide primers 211 and 133, while the mCHERRY fragment was amplified by oligonucleotide primers 210 and 134. RABV-62-H-E-mK-PolyA (Section 2.23.6) was linearised using oligonucleotide primer pairs 113/30 and 135/31. NEB NEBuilder® HiFi DNA Assembly (Section 2.8) was then performed to assemble all PCR fragments. The resulting construct was name RABV-62scFv. Sequences of these primers are listed in Appendix 2.

## 2.24 OPTIMISATION OF VIRUS RESCUE

### 2.24.1 Assessment of the human heavy chain SP

BHK-21 cells were cultured overnight ( $4 \times 10^4$  cells /well) before transfection with 100ng of DNA using FuGENE at a 3:1 ratio. In triplicate, wells were either transfected, according to Section 2.10, with:

1. pNL1.1 sec62-NLuc
2. pNL1.1 62-NLuc
3. pNL1.1 NLuc (Positive control)

After 16 h incubation at 37°C 5% CO<sub>2</sub>, 50 µL media was removed from all wells. This media was then aliquoted into unused wells. This allowed the quantitation of NLuc which had been secreted into the extracellular media and that which still remained inside transfected cells. All measurements were taken as described in Section 2.7. To interpret results, all RLU were normalised and represented as a fold-change from the negative, cell only, control (Cell Blank).

## 2.25 *IN VIVO* STUDIES

### 2.25.1 Mice

BALB/c mice were purchased from registered UK breeder Charles River at three to four weeks of age. Upon arrival, mice were randomised using a random number generator and assigned into groups of four. Within each group, each mouse was marked for identification as either mouse one, two, three, or four. All mice were given food and water *ad libitum* alongside environmental enrichment and checked once a day prior to any experimental procedures, and two to three times a day throughout the course of experiments.

### 2.25.2 Viral Mouse Challenge and termination

Mice were either inoculated by the intravenous (iv), ic, or im route. Virus was delivered in volumes of 30  $\mu$ L. When inoculating virus by the iv route, virus was delivered into the tail vein. When inoculating virus by the im route, virus was delivered into the thigh muscle of both hind legs. When delivering virus through ic inoculation, mice were anaesthetised by inhalation of isoflurane, local anaesthesia administered by the subcutaneous route, and virus administered through the skull by a needle.

After virus inoculation (by any route) mice were observed until the end of the experiment or until mice reached the humane endpoint defined within each study. The scoring system used in these studies is shown in each chapter. Upon reaching the defined humane endpoint, mice were anaesthetised by isoflurane and terminated by cervical dislocation.

### 2.25.3 Serology

Blood samples were taken from mice which reached the defined human endpoint of each study. During anaesthesia, mice were bled directly from the heart by a needle inserted into the chest cavity. Blood was stored at 4 °C until a clot formed where samples were briefly centrifuged (860 x g, 10 min), sera removed and stored at 4 °C. A FAVN test (Section 2.17) was then used to evaluate the presence of anti-RABV antibodies.

### 2.25.4 Formalin fixation of mouse carcasses

After anaesthesia, cardiac bleeding, and cervical dislocation, whole mouse carcass was fixed by submersion in 10% neutral buffered formalin for a minimum of 14 days. To ensure appropriate fixation of all tissues the skull, abdomen, and chest cavity were opened.

## 2.26 HISTOPATHOLOGY

### 2.26.1 Preparation of formalin-fixed mouse carcasses for histopathological analysis

After formalin fixation, mice were removed from the high-containment facility and handed to the Histology unit at the Animal and Plant Health Agency (APHA). Tissues from mice were then removed, decalcified by EDTA, and embedded in paraffin wax. 3-4  $\mu$ m sections were cut from paraffin blocks using the Shandon Finesse ME+ microtome (Fisher) and mounted on positively charged slides.

### 2.26.2 Histopathological staining and imaging

Histopathology was performed by the Histology department at the APHA. Here, sections from mice were fixed in formalin for two weeks before embedding in paraffin wax. 3  $\mu$ m sections were cut and stained for either MAC387 [MCA874, BioRad, (macrophages)], CD3 [A0452, Dako

(T cells)], CD79a [MCA2538 BioRad (B cells)], or RABV N protein [5B12 (MyBioSource)]. All embedding, dewaxing, and staining was performed by the Histology department at the APHA. Images were acquired using a NanoZoomer 2.0RS Slide Scanner at either 100x or 200x magnification.

## 2.27 ANALYTIC SOFTWARE

### 2.27.1 Benchling

Benchling was used in the analysis of all DNA and protein sequences throughout this project. Benchling [Biology Software]. (2017-2021). Retrieved from <https://benchling.com>.

### 2.27.2 NEB Tm Calculator

For PCR reactions using either polymerase Q5 or Q5 Hot Start, oligonucleotide primer T<sub>m</sub> was calculated using the NEB Tm Calculator (<https://tmcalculator.neb.com/>). After inputting the appropriate settings in 'Product Group' and 'Polymerase/Kit', 'Primer Concentration (nM)' was set to 500.

### 2.27.3 NEBaseChanger®

To generate oligonucleotide primers used for all site-directed mutagenesis reactions, NEBaseChanger® (v1.2.9) (<http://nebasechanger.neb.com/>) was used.

### 2.27.4 NEBuilder®

To generate oligonucleotide primers used for all NEBuilder® HiFi Assembly, NEBuilder® (v2.2.7) (<https://nebuilder.neb.com/#!/>) was used. Here, after selecting the appropriate 'Product/Kit' and 'PCR Polymerase/Kit', a minimum overlap of 20 nt, primer concentration of 500 nM, and minimum primer length of 18 nucleotide (nt) was specified.

### 2.27.5 OligoEvaluator™

To assess secondary structure and presence of primer dimers in oligonucleotide primers, Sigma-Aldrich OligoEvaluator™ was used (<http://www.oligoevaluator.com/>).

### 2.27.6 Promega Tm for Oligos Calculator

For PCR reactions using the GoTaq® Green Master Mix kit (M7122), primer T<sub>m</sub> was calculated using the Promega Tm for Oligos Calculator (<https://www.promega.co.uk/resources/tools/biomath/tm-calculator/>). Here, using a primer concentration of 500 nM, primer T<sub>m</sub> was calculated, and PCR performed at 5° below the lower T<sub>m</sub> of the primer pair.

### 2.27.7 SnapGene Viewer

All images of DNA sequences and plasmid maps used throughout this thesis were generated using SnapGene software (from Insightful Science; available at [snapgene.com](http://snapgene.com)).

## 2.28 STATISTICAL ANALYSIS

Data sets were analysed for statistical significance using the GraphPad Prism software. All data sets were analysed for normal (gaussian) distribution and either a Student's t-test, one-way analysis of variance (ANOVA), or Kruskal-Wallis test was selected as appropriate. For ANOVAs, the F test value was also reported. When analysing survival curve graphs for statistical significance, a Log-rank (Mantel-Cox) test was performed. Throughout this thesis, the cut-off for statistical significance was set at a p value of 0.05, with \*  $\leq 0.05$ , \*\*  $\leq 0.01$ , \*\*\*  $\leq 0.001$ , and \*\*\*\*  $\leq 0.0001$ .

## 2.29 ADDGENE

GFP-TNFalpha was a gift from Jennifer Stow (Addgene plasmid # 28089)

## Chapter 3 - VIRUS CLONING AND RESCUE

### 3.1 INTRODUCTION

Over the past decade there has been a substantial increase in the development of live attenuated RABV based vaccines as an alternative to inactivated RABV vaccines. A large proportion of these novel constructs have been created through the insertion of NVE within the RABV genome. Reviewed in Smith et al <sup>180</sup>, these NVEs have been inserted in various positions in the RABV genome and have been consistently effective in attenuating RABV and promoting the development of immunity <sup>180,323</sup>. These developments, in combination with the knowledge that high affinity anti-RABV antibody production localised within the CNS is needed to clear RABV infection <sup>105</sup>, formed the basis of this project: Using a live-attenuated RABV to express a secreted high-affinity anti-RABV neutralising antibody.

scFv antibodies are among the smallest immunoglobulin-based antigen binding molecules, comprising the variable heavy and light chain domains linked by a flexible linker region. Furthermore, scFv are easily linked to purification or labelling tags whilst maintaining binding ability <sup>324</sup>. This makes them ideal for expression within a viral vector. The scFv selected for use, 62-71-3 (binds AS-I of RABV G <sup>302</sup>) was first characterised by Both et al at St. Georges <sup>302</sup>, University of London, UK. Here, 62-71-3 scFv was expressed within a cassette alongside purification/labelling tags (8xHis and E-tag) and a DsRED fluorophore. This cassette was also preceded by a human heavy chain SP promoting secretion into the extracellular environment.

The following chapter describes the process of inserting DNA encoding 62-71-3 scFv within the attenuated RABV vector RABV-cSN and recombinant virus rescue. This involved multiple optimisations including replacement of the DsRED fluorophore with mCHERRY and the removal of the 8xHis and E-tag region. Prior to any attempts at RABV rescue, different methods of T7 polymerase were investigated to optimise RABV rescue. The human heavy chain SP, which precedes the 62-71-3 scFv cassette, was also investigated for its ability to promote secretion of the 62-71-3 scFv in mammalian cell culture.

## 3.2 RESULTS

### 3.2.1 Generation of a Nanoluciferase expressing construct under the control of a T7-promoter

As discussed previously, RABV rescue involves the simultaneous transfection of a full-length RABV plasmid alongside RABV helper plasmids expressing helper proteins N, P, and M <sup>318</sup>. All helper plasmids are under the control of a T7-polymerase (T7) promoter. In an attempt to optimise RABV rescue, alternative methods for T7 expression were assessed. To assess the effectiveness of each method of expressing T7, a Nanoluciferase expressing construct under the control of a T7-promoter was created. Figure 3:1 illustrates the reverse genetics involved in the creation of this construct, named pTIT-NLuc, while sequences of the oligonucleotides used are shown in Section 2.23.1. In short, an NLuc-expressing plasmid (pNL1.1) purchased from Promega (Promega, N1091) was amplified using oligonucleotide primers 61 and 62, while the pTIT plasmid was linearised using oligonucleotide primers 60 and 63. These fragments were then assembled to create plasmid pTIT-NLuc. Figure A1 shows an agarose gel of products from the PCR of NLuc (A) and pTIT-N (B), and the assembled pTIT-NLuc construct (C).

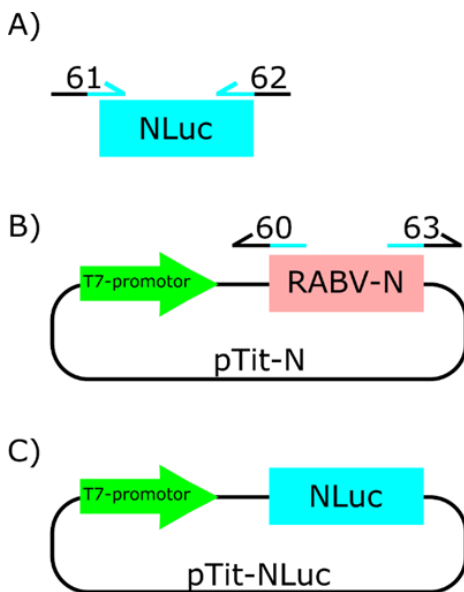


Figure 3:1: Schematic of the cloning process involved in the creation of the pTIT-NLuc vector. First, (A) NLuc was amplified from the pNL1.1.CMV[Nluc/CMV] (Promega, N1091) vector using oligonucleotide primers 61 and 62 while pTIT-N was linearised (B) using oligonucleotide primers 60 and 63. These fragments were then assembled to form pTIT-NLuc. Here, blue sections of oligonucleotide primers indicate homology to NLuc while black sections indicate homology to the pTIT-N vector. The sequences of all oligonucleotide primers shown are listed in Appendix 2.

### 3.2.2 Optimisation of T7-polymerase expression in Virus Rescue

Three methods of eliciting T7 expression in cell culture were investigated; first, the transfection of a plasmid expressing T7 (pTIT-T7) at different timepoints; second, the infection of cell culture with Fowlpox T7 (FP-T7), a FowlPox virus engineered to express T7 upon infection; lastly, through the use of the BSR-T7/5 cell line which, through stable transformation, constitutively expresses T7.

Three timepoints were selected to assess when transfection of pTIT-T7 would elicit the highest expression of T7 for effective virus rescue. pTIT-T7 was transfected (Section 2.10) at either six or three h before transfection of pTIT-NLuc, or at the time of pTIT-NLuc transfection (0 h). To assess whether infection of cell culture with FP-T7 could result in high levels of T7 being expressed, BHK-21 cell culture was infected with a multiplicity of infection (MOI) ranging from one to five for one h (Section 2.11) before removal of media and transfection with pTIT-NLuc. Lastly, BSR-T7/5 cells were seeded alongside BHK-21 cells. To interpret results, all RLU were normalised and represented as a fold-change from the negative, cell only, control.

As shown in Figure 3:2, there was no statistically significant difference between FowlPox infection of BHK-21 cell culture at MOIs from one to five. In contrast, a significant difference (\*\*\*\*) between NLuc expression in BHK-21 cells transfected either 6 h before or at the time of pTIT-NLuc transfection was seen. However, FowlPox infection at a MOI of 5 and transfecting pTIT-T7 alongside pTIT-NLuc both resulted in significantly lower RLU values than BSR-T7/5 cells.

In all subsequent RABV rescue experiments, BSR-T7/5 cells were used as these were shown to express the highest levels of T7 polymerase, allowing the highest expression of RABV helper plasmids (N, P, and L) and the full-length RABV construct plasmid.

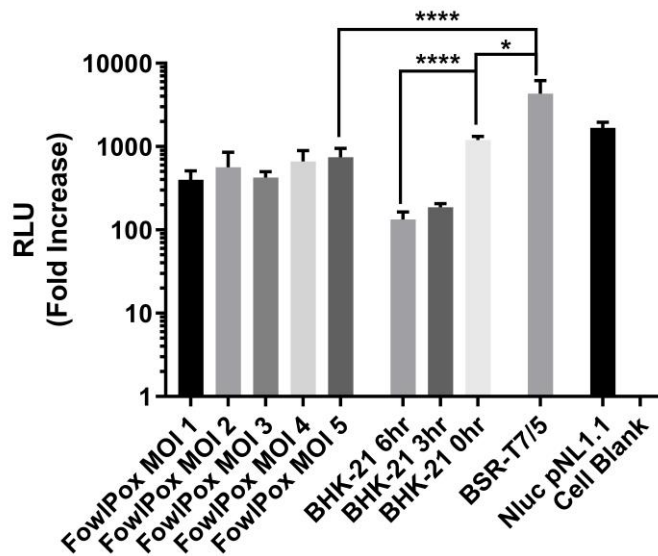


Figure 3:2: Investigation of the optimal method of enabling T7 polymerase expression in mammalian cell culture. All luciferase values have been displayed in RLU as a fold change increase from the negative control (cell blank). Here, a Kruskal-Wallis test was used to show statistical significance where \*\*\*\*  $P \leq 0.0001$  and \*  $P \leq 0.1$ .

### 3.2.3 Generation of a nanoluciferase construct to assess the ability of a human heavy chain SP to promote secretion in BSR-T7/5 cell culture.

To assess the ability of the human heavy chain SP to promote secretion into extracellular media two constructs were created. These constructs were based upon the pNL1.1 vector described in Section 3.2.1 where the 62-71-3 scFv cassette (without the DsRED) had been inserted, either with or without the SP (pNL1.1 sec62-NLuc and pNL1.1 62-NLuc). Section 2.23.2 describes the cloning procedure used to generate these constructs. A schematic is shown in Figure 3:3. This involved the creation of three PCR products (55/56, 59/56, and 58/57), which were assembled to create pNL1.1 sec62-NLuc and pNL1.1 62-NLuc. All PCR products and resulting assembled constructs were separated on an electrophoresis gel and are shown in Figure A2.

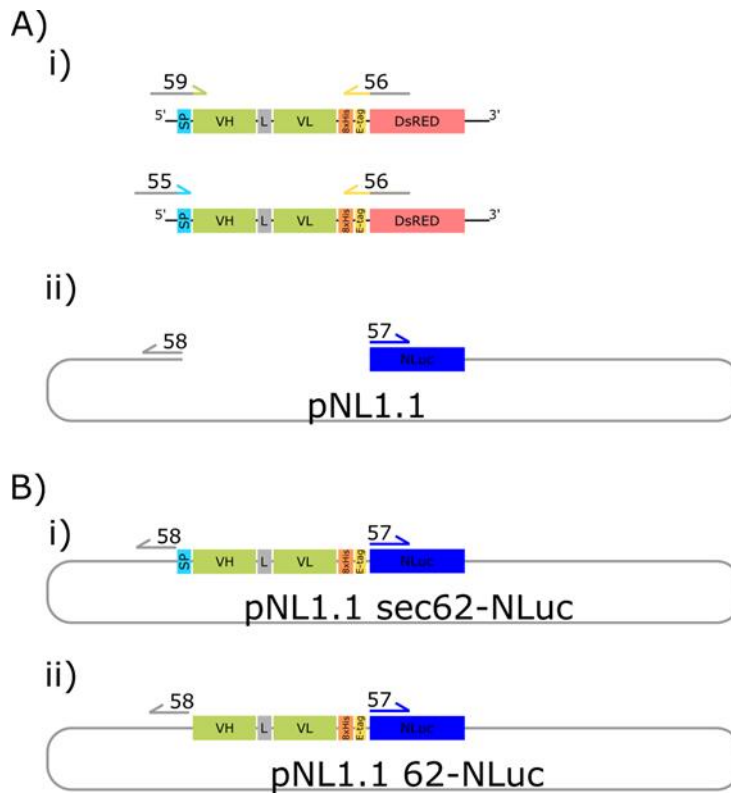


Figure 3:3: Schematic illustrating the cloning involved in the production of pNL1.1 sec62-NLuc and pNL1.1 62-NLuc. After linearization of the 62-71-3 scFv (including 8xHis and E-tag) using primers 55/59 and 56 (Ai) and the linearization of the NLuc-containing pNL1.1 vector (Aii), fragments were assembled to form two pNL1.1 constructs. First, (Bi) contained the secreted form (containing the SP) of 62-71-3 scFv linked to both 8xHis and E-tag, and the NLuc. Second, (Bii) contained the non-secreted form (no SP) of 62-71-3 with all aforementioned His/E/NLuc components.

### 3.2.4 Assessment of the human heavy chain SP in promoting secretion from mammalian cell culture

To assess the ability of the SP to promote secretion of 62-71-3, the two constructs generated in Section 3.2.3 (pNL1.1 sec62-NLuc and pNL1.1 62-NLuc) were used. As described in Section 2.24.1, BHK-21 cells were cultured overnight and transfected with either:

4. pNL1.1 sec62-NLuc
5. pNL1.1 62-NLuc
6. pNL1.1 NLuc (Positive control)

After 16 h, luciferase signal was measured from cell lysate and supernatant. All measurements were taken as described in Section 2.7. To interpret results, RLU were normalised and represented as a fold-change from the negative cell only control (Cell Blank).

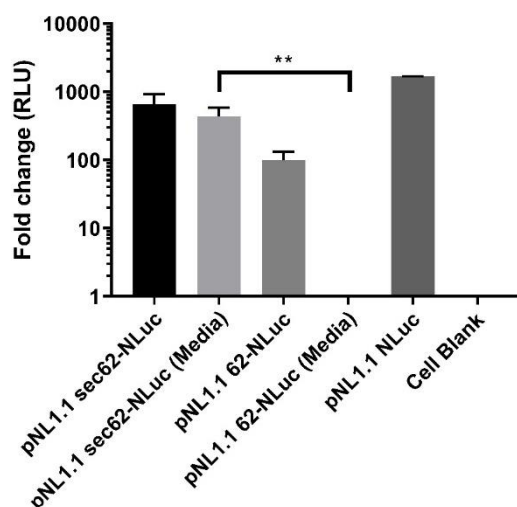


Figure 3:4: Investigating the effectiveness of the human heavy chain SP sequence in the secretion of 62-71-3 ScFv from BHK-21 cell culture. Here, two pNL1.1 vectors expressing nanoluciferase were constructed. In these vectors, a human heavy chain SP sequence was either present upstream of nanoluciferase (pNL1.1 sec62-NLuc) or was absent (pNL1.1 62-NLuc). Nanoluciferase was measured in either cell lysate or cell culture media. pNL1.1 NLuc (nanoluciferase) has been included as a positive control, while 'cell blank' has been included as a negative control. All luciferase values have been displayed in RLU as a fold change increase from the negative control (cell blank). Data has been averaged between repeats (n=3), with error bars representing the standard error of the mean. Statistical comparison was performed using unpaired Students t-test, with  $^{**}P \leq 0.01$ .

As shown in Figure 3:4, high amounts of NLuc were detected in the extracellular media of cells transfected with pNL1.1 sec62-NLuc, but not pNL1.1 62-NLuc. These data illustrated that, while a proportion of NLuc remained within cells despite the presence of the SP, this SP was effective in promoting secretion from BHK-21 cells. Therefore, this SP was kept in all further secreted 62-71-3 scFv constructs.

### 3.2.5 Insertion, mutation, and replacement of elements within RABV-cSN for successful virus rescue

#### 3.2.5.1 Insertion of the 62-71-3 scFv cassette within the RABV genome

The 62-71-3 scFv cassette is illustrated in Figure 3:5A. This comprises the 62-71-3 scFv linked to an 8xHis tag, an E-tag, and a DsRED fluorophore for visualisation in cell culture. As described in Section 2.23.3 and illustrated in Figure 3:5, both the 62-71-3 scFv cassette and RABV-cSN backbone were linearised and assembled to create construct RABV-62-H-E-DsR.

The oligonucleotide primers used to amplify the 62-71-3 scFv cassette (34 and 3) contained regions homologous to both the 62-171-3 scFv cassette (5' end) and the RABV-cSN backbone (3' end). However, to enable expression of the cassette the transcriptional start unit and stop unit from the RABV P gene was incorporated before and after the 62-71-3 scFv cassette respectively\*. Gene borders from the RABV P gene were selected based on communications regarding previous, successful, virus rescues. Figure A3 shows the PCR products involved in the insertion of the 62-71-3 scFv cassette (including the 8xHis tag, E-tag, and DsRED) into the cSN backbone, including the resulting construct, RABV-62-H-E-DsR.

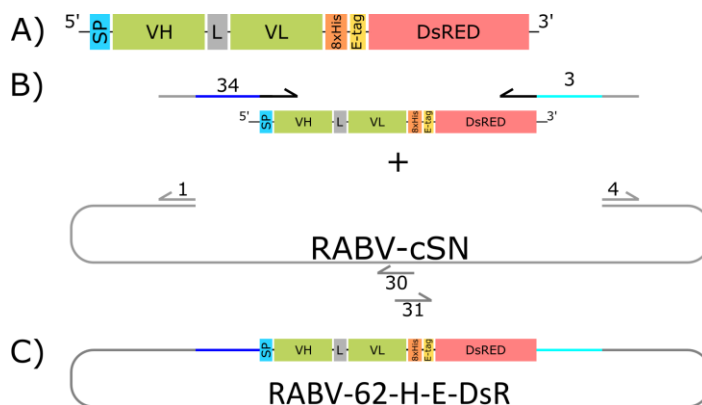


Figure 3:5: A schematic showing the 62-71-3 scFv cassette and its subsequent cloning strategy into the RABV-cSN backbone vector. A) a schematic of the 62-71-3 scFv cassette. B) the oligonucleotides involved in the amplification of the scFv cassette and linearization of the RABV-cSN vector. Oligonucleotide primers 3 and 34 were used to amplify the scFv cassette and incorporate transcriptional start (34) and stop (3) sequences to the beginning and end of the scFv cassette.

#### 3.2.5.2 Replacement of DsRED with mCHERRY for enhanced cell culture visualisation

DsRED is a fluorescent protein linked to the 3' end of the 62-71-3 scFv cassette with an excitation and emission wavelength of 558 and 583 nm, respectively. However, as previously

reported, DsRED has many shortcomings including slow maturation time and obligate tetramerisation<sup>325,326</sup>. For these reasons, DsRED was substituted for mCHERRY within the 62-71-3 scFv cassette. To perform this substitution, as described in Section 2.23.4 and shown in Figure A4, mCHERRY (including the preceding Kozak sequence) was amplified from the pRVdG-mCHERRY-N-P-M-EGFP-SynPhRFP-L plasmid (Addgene: 52481) and used to replace the DsRED of RABV-62-H-E-DsR. The PCR products involved in this substitution are shown in Figure 3:6 alongside the fully assembled construct which was named RABV-62-H-E-mCH.

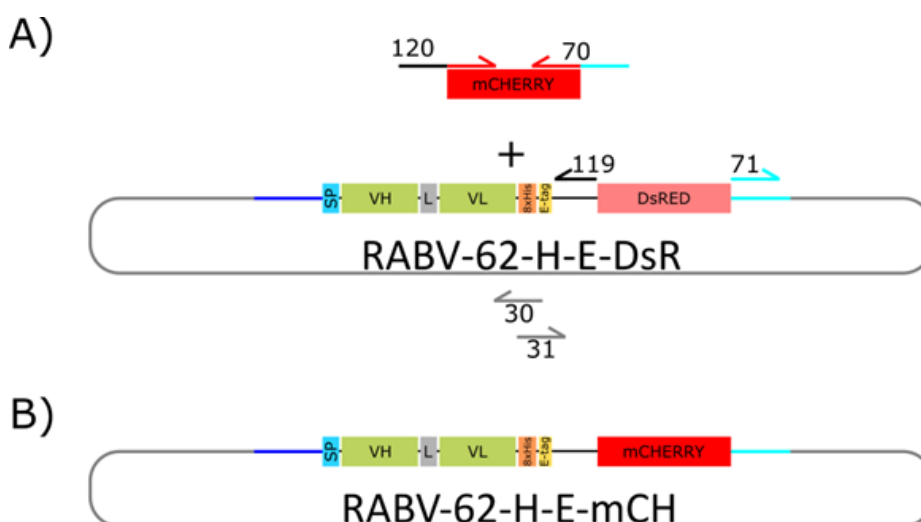


Figure 3:6: A schematic of the cloning strategy used to substitute the DsRED fluorophore for the mCHERRY fluorophore to enhance visualisation of infected cells in cell culture. (A) After the amplification of mCHERRY from Addgene #52481 using oligonucleotides 120 and 70, the RABV-62-H-E-DsR backbone was linearised in two parts by oligonucleotide primer pairs 119/30, and 71/ 31. The PCR products from these PCR reactions were then purified and assembled to form the RABV-62-H-E-mCH construct shown in (B). All oligonucleotides have been colour coded to their homologous regions where; red indicates binding to mCHERRY, black indicates binding to the 62-71-3 scFv cassette, light blue indicates binding to the transcriptional stop sequence after DsRED, and grey indicates binding to the RABV-62-H-E-DsRED backbone. The sequences of all oligonucleotide primers shown are listed in Appendix 2.

**3.2.5.3 Mutation of RABV-cSN G at site 226 to prevent self-neutralisation by 62-71-3 scFv**

62-71-3 has been previously demonstrated to target AS-I of the RABV G protein. Specifically, amino acid 226 had been identified where mutating K to an R was sufficient to reduce the neutralisation of RABV G by 62-71-3 by approximately 75 %<sup>302</sup>. Therefore, to diminish self-neutralisation by 62-71-3 scFv of the host RABV, a K226R mutation of the RABV G gene within the full-length construct was performed (schematic shown in Figure 3:7). Due to the large size

of the RABV-cSN vector, site-directed mutagenesis was performed on the glycoprotein gene in the pTIT-G vector (~5 kb) (Figure A5). The pTIT-G vector based upon the same pTIT backbone as the pTIT-N/P/L plasmids used in RABV rescue (Section 2.12.1) but contains the RABV glycoprotein.

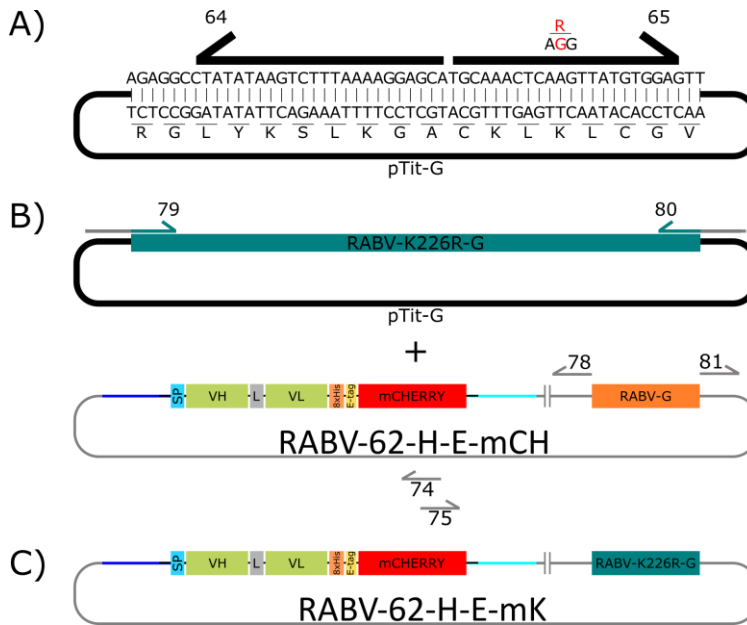


Figure 3:7: A schematic illustrating the cloning strategy employed to induce a K226R mutation in the RABV glycoprotein gene of the pTIT-G vector, and its subsequent substitution into a linearised RABV-62-H-E-mCH vector. (A) shows the binding sites of the two oligonucleotides used to induce the K226R mutation in the RABV glycoprotein gene of the pTIT-G vector (64 and 65). Here, as illustrated in red the codon encoding a lysine has been mutated from AAG to AGG to encode an arginine. After site directed mutagenesis of K226R within the RABV G gene of pTIT-G, (B) RABV-K226R-G was amplified using oligonucleotide primers 79 and 80. The RABV-62-H-E-mCH backbone was then linearised by oligonucleotide primers 78/74 and 81/75 and all fragments assembled to form (C) RABV-62-H-E-mK. All oligonucleotides have been colour coded to their homologous regions where black indicates homology to RABV G gene, dark green to RABV-K226R-G, and grey to the RABV-62-H-E-mCH backbone. The sequences of all oligonucleotide primers shown are listed in Appendix 2.

Figure A5 shows an agarose electrophoresis gel of all PCR products used including the point mutation of pTIT-G, the linearisation of the RABV G gene from pTIT-G, and its substitution into RABV-62-H-E-DsR to form RABV-62-H-E-mK.

#### 3.2.5.4 Alteration of the gene borders surrounding the 62-71-3 scFv cassette within RABV

In Section 3.2.5.1, the 62-71-3 scFv cassette was inserted into the RABV-cSN backbone. During this, transcriptional start and stop units were inserted before and after the scFv cassette. Unfortunately, due to errors in identifying the correct borders of the transcriptional start and stop units, some of the sequences within the designed oligonucleotide primers were either absent or incorrect. Within oligonucleotide primer 34, the transcriptional start sequence inserted upstream of the scFv cassette which was thought to only contain the RABV P gene transcriptional start sequence, also contained the Poly(A) tail sequence from RABV N. Furthermore, oligonucleotide primer 3 (which inserted the RABV P gene transcriptional stop sequence) did not include a Poly(A) tail necessary for termination transcription of the upstream scFv cassette.

As described in Section 2.23.6 and illustrated in Figure 3:8, the 62-71-3 scFv cassette insert was re-cloned into the same position in RABV-H-E-mK. While the insert position (before the N gene) was kept constant, the transcriptional start and stop units were altered to match that of the positive control virus, RABV-EBLV-1 (*European bat Lyssavirus 1*)-eGFP (Guanghui Wu, APHA personal communication). These alterations include the removal of the RABV N gene Poly(A) tail upstream of the scFv cassette and the incorporation of a Poly(A) tail in the transcriptional stop unit present after the scFv cassette which is then followed by an effective RABV N gene transcriptional start sequence. The PCR products involved in this cloning procedure, as described in Figure 3:8, are shown in Figure A6 alongside the fully assembled product RABV-62-H-E-mK-Poly(A).

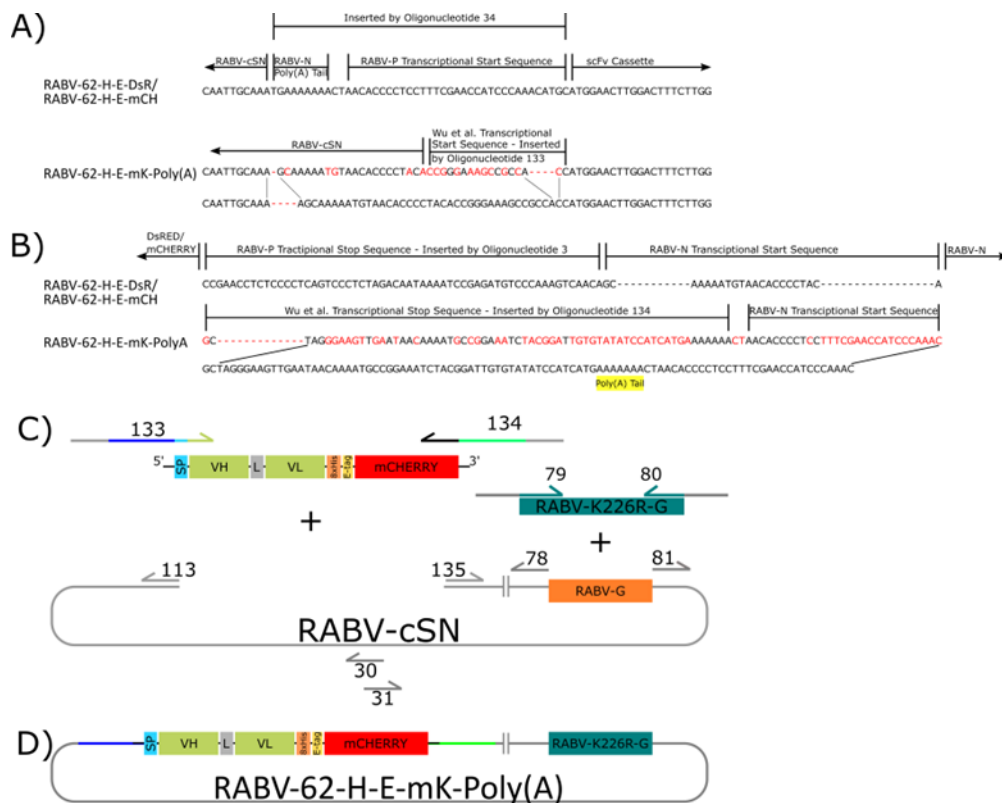


Figure 3:8: The cloning method employed for the re-insertion of the 62-71-3-mCHERRY cassette into the cSN (K226R-G) backbone. First, the pre- (A) and post- (B) transcriptional sequences inserted before and after the scFv cassette by oligonucleotides 34 and 3 respectively were changed to match that of the positive control virus for virus rescues, RABV-EBLV-1-eGFP. Here (A and B), oligonucleotide primer pairs 34/133 and 3/134 have been aligned to RABV-62-H-E-DsR/mCH where bases highlighted in red indicate a change and '-' indicates an insertion or deletion. In A, the RABV N gene Poly(A) sequence upstream of the scFv cassette has been removed. In B, a functional poly(A) tail has been added and leads to a functioning gene border with RABV N gene. C, shows the cloning strategy used to incorporate the scFv cassette with new transcriptional start and stop units into the RABV-cSN vector. Furthermore, the RABV G gene has also been substituted for a RABV-K226R-G gene. Together, these fragments are assembled to form the vector named RABV-62-H-E-mK-PolyA. All oligonucleotides have been colour coded to match their homologous regions where dark blue encodes the transcriptional start unit, light blue to the SP, light green to transcriptional stop unit, dark green to RABV-K226R-G, and grey to the RABV-cSN backbone. The sequences of all oligonucleotide primers shown are listed in Appendix 2.

### 3.2.6 Virus rescue of RABV-62-H-E-mk-Poly(A)

To this point, all mutations deemed necessary for the viral rescue of 62-71-3 scFv-expressing RABV had been performed. Furthermore, each mutation had been confirmed by Sanger sequencing (Section 2.5.1) of the mutation sites themselves and the vector-insert borders.

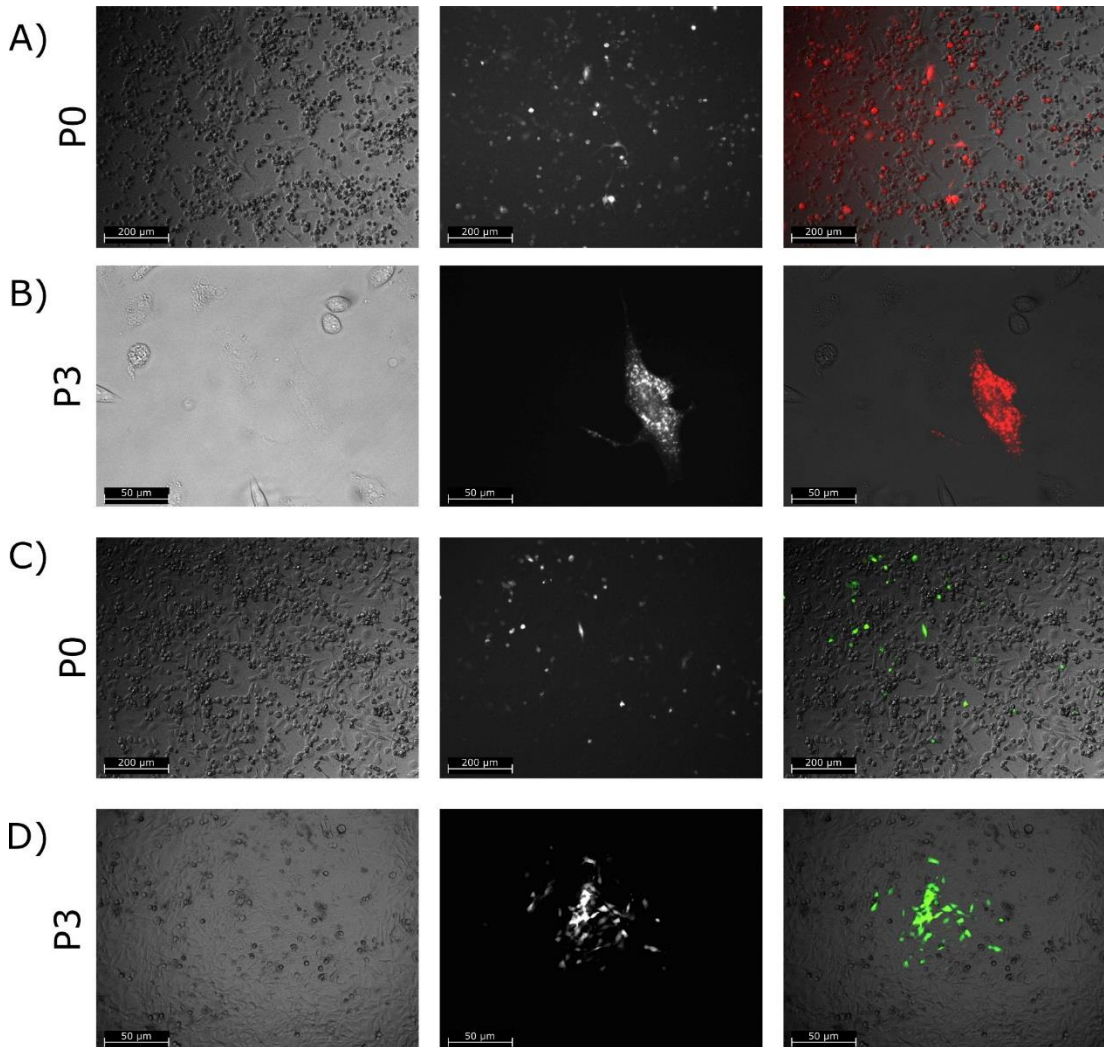


Figure 3:9: Images showing viral rescue of RABV-62-H-E-mK-Poly(A) and RABV- EBLV-1-eGFP. All images are displayed as bright-field images of cell culture cells, UV images of fluorescence, and a merged (bright-field+UV) image. Row A shows RABV-62-H-E-mK-Poly(A) rescue cells at passage 0, while row B shows passage three. Row C shows RABV- EBLV-1-eGFP rescue cells at passage 0, while row D shows passage three.

As shown in Figure 3:9, RABV-62-H-E-mk-Poly(A) and the positive control virus RABV-EBLV-1-eGFP were taken forward to virus rescue. After transfection of RABV-62-H-E-mk-Poly(A) with the necessary helper plasmids into BSR-T7/5 cell culture, fluorescence was observed in cells under UV at passage 0 (Figure 3:9A). However, after multiple passages (Figure 3:9B) the

pattern of intracellular fluorescence became increasingly clumped, rather than uniformly distributed, and cell death quickly followed. Interestingly, single-cell fluorescence could be observed up to passage 10, where the experiment was terminated, but fluorescence did not spread to neighbouring cells to form foci. In contrast, RABV- EBLV-1-eGFP displayed typical signs of virus rescue. Three days after transfection at passage 0 (Figure 3:9C), cells were uniformly fluorescent under UV and their morphology did not suggest progression into cell death. Furthermore, after multiple passages to passage three (Figure 3:9D), this fluorescence and phenotype was maintained, and fluorescent foci were observed to be growing over time.

While the fluorescence observed within RABV-62-H-E-mk-Poly(A) rescue cells at passage 0 was thought to arise from transient transfection, fluorescence present after multiple passages was believed to be from RABV-62-H-E-mk-Poly(A) virus infection. From observations of cell morphology over time, cells displayed a phenotype which led quickly to cell death before sufficient virus numbers could be produced to form foci. To confirm the presence of virus, RNA was extracted from cell culture media (at passage 10) and RT-PCR performed using oligonucleotide primers (79 and 80) specific for the 62-71-3 scFv mCHERRY cassette. Figure 3:10 shows the presence of 62-71-3 scFv RNA in the tissue culture supernatant of virus rescue cell cultures.

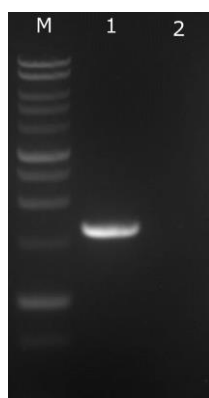


Figure 3:10: 1% agarose gel showing a DNA band from RT-PCR of virus RNA extraction. After TriZOL extraction of RNA from virus culture supernatant, RT-PCR was performed using specific 62-71-3 scFv oligonucleotide primers 246 and 247 (expected size 1575 bp) to probe for the presence of RABV produced 62-71-3 scFv. M: ladder, lane 1: RABV-62-H-E-mK-Poly(A) TriZOL extraction RT-PCR, lane 2: RABV-cSN TriZOL extraction RT-PCR.

### 3.2.7 Investigating the failure of virus rescue

As discussed above, multiple attempts to rescue RABV-62-H-E-mK-Poly(A) were unsuccessful. While RT-PCR confirmed the presence of RABV in cell culture supernatant, phenotypic analysis

of infected cells revealed a consistent pattern of phenotypically healthy cells fluorescing under UV before cells displayed an aberrant phenotype which quickly progressed to cell death. Furthermore, this pattern of cellular fluorescence transitioning into cell death could be maintained over multiple passages. This suggests that while transfection of full-length RABV-62-H-E-mK-Poly(A) (and accompanying helper plasmids) was successful in producing infectious and replicating virus, RABV-62-H-E-mK-Poly(A) was inducing cell death after a short period of replication (where infectious virus could be released).

Four potential causes of this apoptotic phenotype were identified and investigated:

1. The cause of the apoptotic phenotype could be cell-type specific. Could attempting virus rescue in other cell lines abolish the development of this phenotype.
2. The K226R mutation induced in Section 3.2.5.3 is allowing some residual binding of 62-71-3 to RABV G protein which may be causing cell death.
3. The development of the apoptotic phenotype and cell death is due to the 62-71-3 scFv gene.
4. The 8xHis-tag and E-tag elicit an apoptotic phenotype which progresses to cell death within mammalian cell culture.

#### 3.2.7.1 Is the cell death caused by RABV-62-H-E-mK-Poly(A) cell type specific?

As discussed in Section 3.2.3, BHK-21 cells were exchanged for BSR-T7/5 cells for enhanced T7-polymerase expression. To investigate whether the apoptotic phenotype depicted in Figure 3:9, was cell culture line dependent, virus rescue was performed in multiple different cell lines; BHK-21, BSR-T7/5, CHO, HEK-293T, and N2a. These cell lines were chosen due to their use in previous attempts to rescue rabies virus variants or in the expression of scFv proteins<sup>17,327-330</sup>.

RABV rescue was performed as described in Section 2.12.1. In cell lines where T7-polymerase was not constitutively expressed (BHK-21, N2a, CHO, and HEK-293T), transient transfection of pTIT-T7 was used as a source of T7 polymerase. In all cell lines red fluorescence was visible under UV light at passages 0 and two to three days after transfection (data not shown). However, this fluorescence did not persist over multiple passages and all fluorescent cells quickly progressed into cell death. In contrast, the positive control RABV- EBLV-1-eGFP could be consistently rescued in all cell lines used and fluorescence maintained over passages. Taken together, these findings indicate that the apoptotic phenotype and cell death observed is not cell line dependent.

### 3.2.7.2 Is residual binding of RABV G protein by vp62-71-3 the cause of cell death?

During the development and characterisation of 62-71-3 scFv, aa 226 of the RABV G was shown to be critical for the neutralisation of RABV G protein by 62-71-3 scFv<sup>302</sup>. Specifically, if residue 226 was mutated from a K to R, neutralisation of a RABV G gene-containing pseudovirus was reduced by approximately 75%<sup>302</sup>. However, this remaining 25% neutralisation capacity might allow residual binding of 62-71-3 to RABV G protein, providing a possible explanation to the granular fluorescence phenotype observed under UV in Section 3.2.6. Taken together, it was decided to evaluate whether the substitution of the RABV G gene for the G gene of a highly divergent Lyssavirus would abolish any residual 62-71-3 scFv binding and development of apoptotic phenotype. The divergent IKOV strain was selected for replacement of the RABV G gene. The ability of 62-71-3 scFv to bind and neutralise IKOV was first assessed by two methods before the IKOV G gene was used to replace the RABV G gene in the RABV-62-H-E-mK-Poly(A) construct. The first method assessed binding of *N. benthamiana* plant-produced 62-71-3 scFv (linked to DsRED, referred to from here on as 62-71-3 scFv DsRED)<sup>308</sup> to a BHK-21 monolayer infected with either IKOV or CVS-11. As shown in Figure 3:11, 62-71-3 scFv DsRED produced in *N. benthamiana* was able to effectively bind and label cells infected with CVS-11, but was not able to bind cells infected with IKOV.

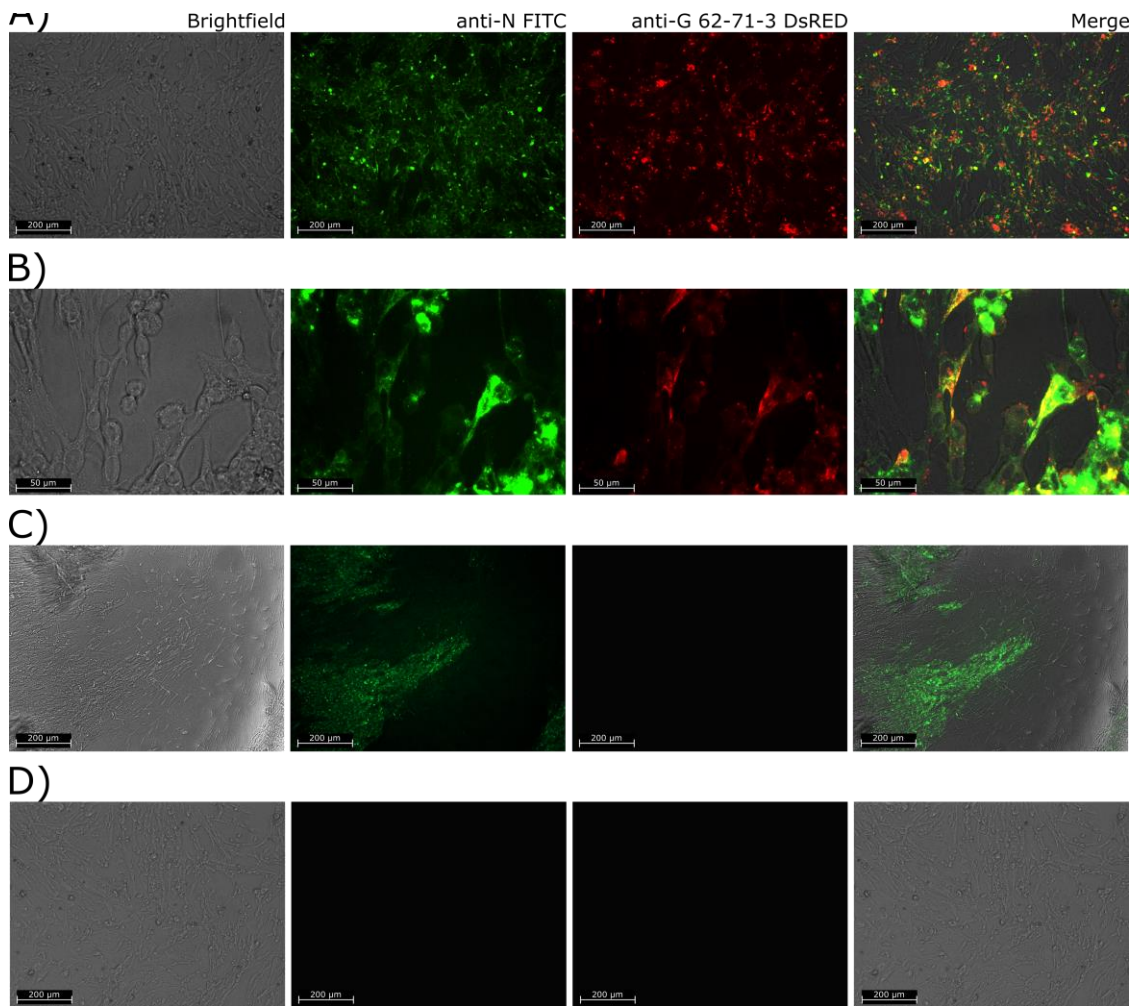


Figure 3:11: BHK-21 cells infected with either CVS-11 (A/B), IKOV (C), or uninfected (D). All cells have been fixed with 80% acetone and incubated with both an anti-N FITC antibody and 62-71-3 DsRED produced in *N. benthamiana* plants. Each column has been labelled to indicate whether cells are shown in brightfield, stained with anti-RABV N protein FITC antibody (Fujirebio), stained for RABV G protein with 62-71-3 scFv DsRED produced in *N. benthamiana*, or are displayed as a merge of all three images.

It was then decided to assess the ability of *N. benthamiana*-produced 62-71-3 scFv DsRED to neutralise live CVS-11 and IKOV by FAVN. The results of this assay are shown in Table 3:1. As shown, 62-71-3 scFv DsRED produced in *N. benthamiana* was effective in neutralising CVS-11, but not IKOV.

| Challenge Virus | Sera assessed for neutralisation | Neutralising/non-neutralising | Reciprocal titre / IU/mL |
|-----------------|----------------------------------|-------------------------------|--------------------------|
| CVS-11          | 62-71-3 scFv                     | Neutralising                  | 43740 / 1066.02          |
| CVS-11          | Cell Culture Media Control       | Not Neutralising              | n/a                      |
| IKOV            | 62-71-3 scFv                     | Not Neutralising              | n/a                      |
| IKOV            | Cell Culture Media Control       | Not Neutralising              | n/a                      |

Table 3:1: Assessment of the neutralising activity of 62-71-3 scFv DsRED (produced by *N. benthamiana*) to both CVS-11 and IKOV.

### 3.2.7.3 Replacement of the RABV G gene of RABV-cSN with the IKOV G gene

As discussed, after demonstrating that that 62-71-3 scFv was unable to bind or neutralise IKOV G protein (Section 4.2.6), the IKOV G gene was used to substitute the RABV G gene within RABV-62-H-E-mK-Poly(A) (Section 2.23.7) and the resulting construct was named RABV-62-H-E-mCH-IKOV (Figure 3:12). The PCR products used in the creation of RABV-62-H-E-mCH-IKOV, as well as assembled RABV-62-H-E-mCH-IKOV, are shown in Figure A7.

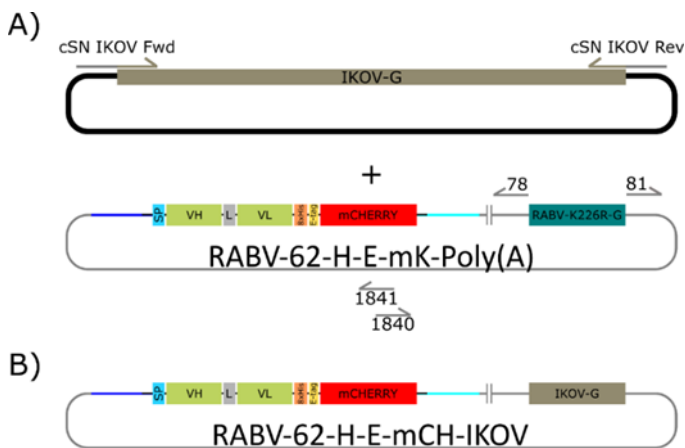


Figure 3:12: Schematic illustrating the cloning method employed when substituting RABV-K226R-G for the IKOV G gene. A, After the amplification of IKOV G gene using oligonucleotide primers 'cSN IKOV Fwd' and 'cSN IKOV Rev', the RABV-62-H-E-mK-Poly(A) vector was linearised in two parts by oligonucleotide primer pairs 78 and 1841, and 81 and 1840. After the assembly of all fragments RABV-62-H-E-mCH-IKOV (B) was created and taken forward to virus rescue. All oligonucleotide primers are colour coded to match homologous regions where brown indicates homology to IKOV G gene and grey to RABV-62-H-E-mK-Poly(A) backbone. The sequences of all oligonucleotide primers shown are listed in Appendix 2.

All attempts at RABV-62-H-E-mCH-IKOV virus rescue in BSR-T7/5 cells were unsuccessful. While fluorescence was present in cell culture three to four days after transfection, this fluorescence

could not be maintained over multiple passages (data not shown). This result is in contrast to the positive control virus for rescue, RABV- EBLV-1-eGFP where fluorescence was present both after initial transfection and after subsequent passage. In summary, these data suggest that binding of 62-71-3 scFv to the RABV G protein was not the cause of the apoptotic phenotype and cell death observed in RABV-62-H-E-mK-Poly(A) rescue experiment. It is important to note, however, that the functionality of the IKOV protein was not assessed and this may have been the cause RABV-62-H-E-mCH-IKOV virus rescue failure. This is an area which should be investigated further in future studies.

#### 3.2.7.4 Is the cause of the apoptotic phenotype and cell death due to the 62-71-3 scFv protein?

To investigate whether the 62-71-3 scFv was itself responsible for the development of the apoptotic phenotype and cell death, possibly due to inappropriate binding of intracellular components, the scFv gene was replaced with a control DNA construct, encoding TNF $\alpha$ . TNF $\alpha$  was selected due to its use in previous research conducted by Faber et al <sup>253</sup> where it was used to attenuate a rabies vaccine-strain vector SPBN. TNF $\alpha$  was able to attenuate SPBN through inhibition of neuronal virus replication and activation of the microglial immune response promoting clearance from the CNS. In this study, two TNF $\alpha$ -expressing RABVs of interest were created (illustrated in Figure 3:13). In the first, full length TNF $\alpha$  was inserted into SPBN. In the second, sequences enabling the secretion of TNF $\alpha$  from host cells were removed before insertion into SPBN. Both of these forms of TNF $\alpha$  were used to substitute the 62-71-3 scFv gene within the RABV-62-H-E-mK-Poly(A) vector.

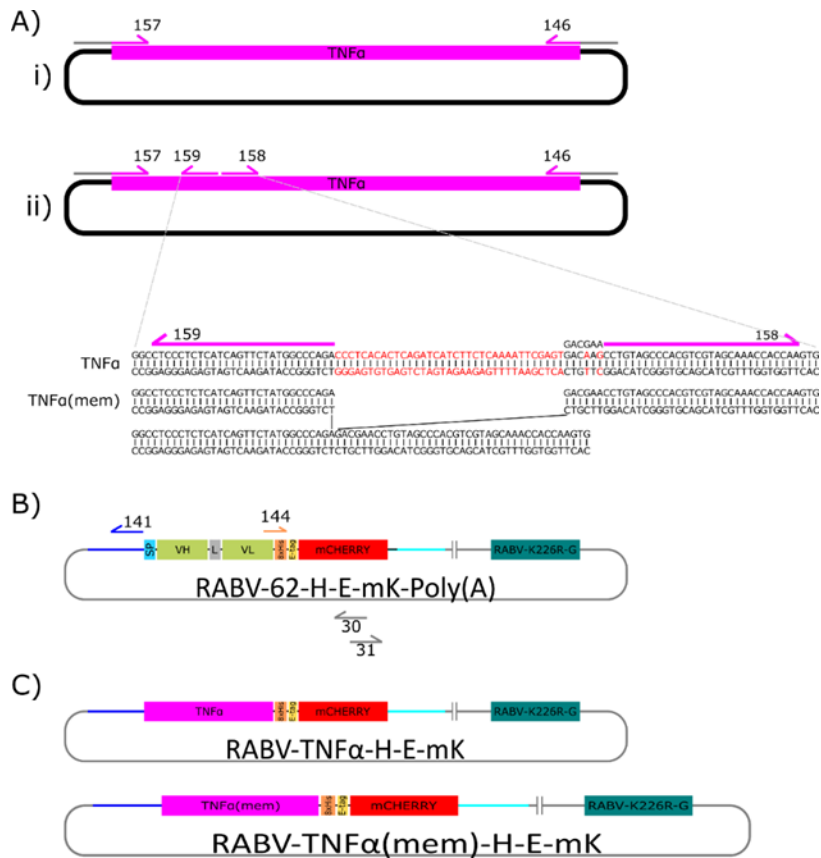


Figure 3:13: A schematic of the cloning used to create two TNF $\alpha$ -expressing RABV constructs, RABV-TNF $\alpha$ -H-E-mK and RABV-TNF $\alpha$ (mem)-H-E-mK. To create RABV-TNF $\alpha$ -H-E-mK, full-length TNF $\alpha$  was amplified using oligonucleotide primer pair 157 and 146 (Ai) and RABV-62-H-E-mK-Poly(A) linearised using oligonucleotide primer pairs 141/30, and 144/31 (B). These fragments were then assembled to create RABV-TNF $\alpha$ -H-E-mK (C). To create membrane-bound TNF $\alpha$  (TNF $\alpha$ (mem)) needed for RABV-TNF $\alpha$ (mem)-H-E-mK, bases critical for the post-translational cleavage of TNF $\alpha$  to allow secretion were removed by site-directed mutagenesis using oligonucleotide primer pair 159 and 158 (Aii). TNF $\alpha$ (mem) was then amplified using oligonucleotide primer pair 157 and 146 (Aii) and substituted into linearised RABV-62-H-E-mK-Poly(A) (B) to form RABV-TNF $\alpha$ (mem)-H-E-mK (C). All oligonucleotide primers have been colour coded to indicate homologous regions where purple indicates homology to TNF $\alpha$ , dark blue to the transcriptional stop unit, orange to the 8xHis tag, and grey to the RABV-62-H-E-mK-Poly(A) backbone. The sequences of all oligonucleotide primers shown are listed in Appendix 2.

Section 2.23.8 and describe the cloning process involved in the creation of RABV-TNF $\alpha$ -H-E-mK and RABV-TNF $\alpha$ (mem)-H-E-mK. In brief, 62-71-3 scFv of the RABV-62-H-E-mK-Poly(A) construct was substituted for either full-length TNF $\alpha$  (Addgene plasmid # 28089) or membrane-bound

TNF $\alpha$  (where the cleavage site had been removed). Figure A8 shows all amplified PCR products, and the assembled constructs, used.

All attempts at RABV-TNF $\alpha$ -H-E-mK and RABV-TNF $\alpha$ (mem)-H-E-mK virus rescue were unsuccessful. After transfection of RABV-TNF $\alpha$ -H-E-mK or RABV-TNF $\alpha$ (mem)-H-E-mK into BSR-T7/5 cell culture, cellular fluorescence under UV was initially visible. However, after multiple passages, cells developed granular fluorescence and quickly progressed to cell death. Furthermore, no fluorescent foci were formed (Figure 3:14A/B).

This contrasts with RABV- EBLV-1-eGFP, the positive control for virus rescue, which was consistently rescued successfully. Here, fluorescence was present after initial transfection (Figure 3:14C) and after multiple passages fluorescent foci were visible (Figure 3:14Ci). To show intracellular TNF $\alpha$  expression did not cause cell death, the TNF $\alpha$ -GFP vector was transiently transfected into BSR-T7/5 cells (Figure 3:14D). Under UV, fluorescent cells did not progress into cell death, suggesting that the granular fluorescence and cell death displayed in fluorescent virus rescue cells was not due to the expression of TNF $\alpha$  (observed through fluorescence of the GFP-TNF $\alpha$  polyprotein).

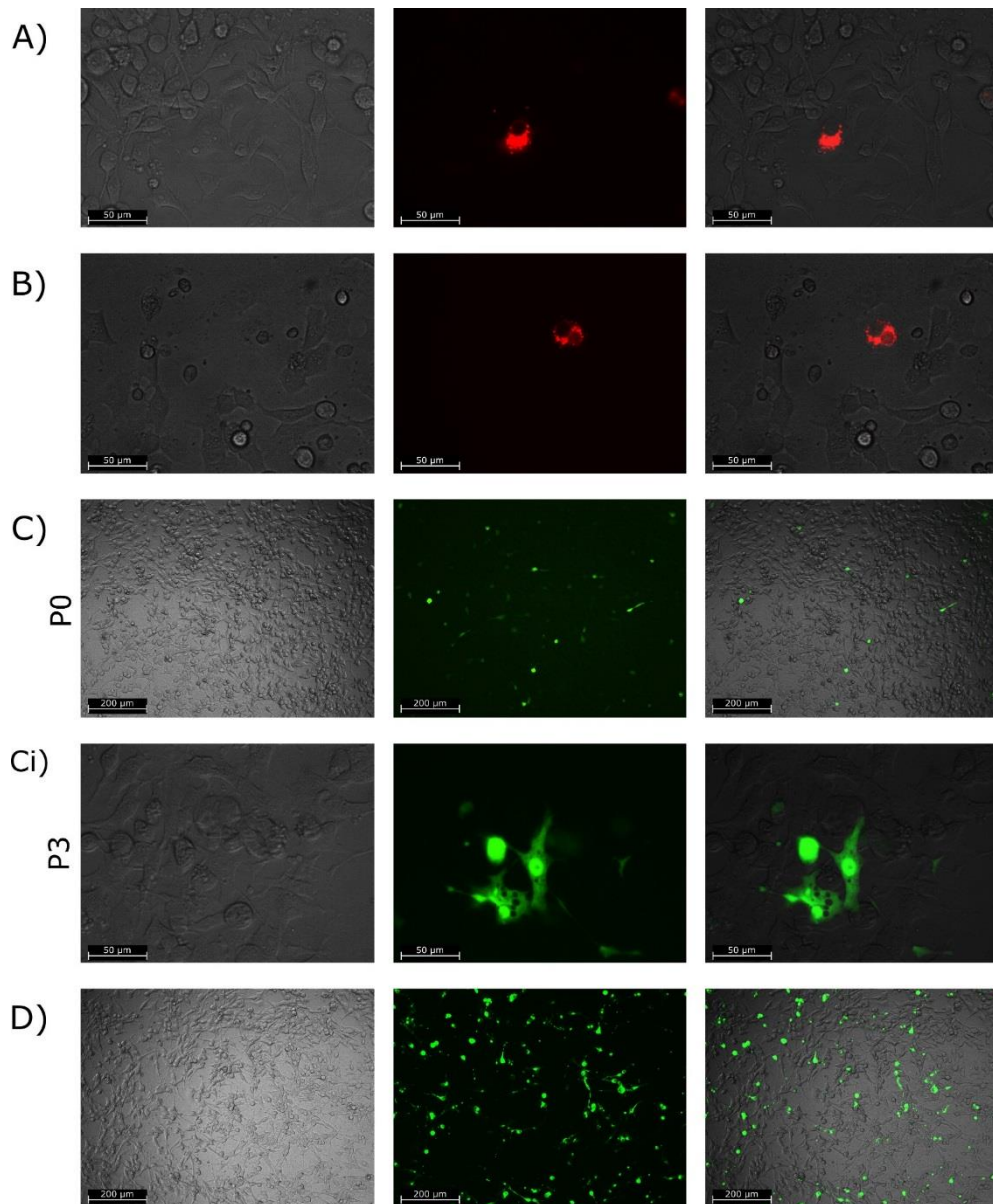


Figure 3:14: UV microscope images of RABV-TNF $\alpha$ -H-E-mK and RABV-TNF $\alpha$ (mem)-H-E-mK virus rescues. From left to right, columns show bright-field images, UV images, and merged (bright-field+UV) images. A and B, shows images taken at passage three after transfection of RABV-TNF $\alpha$ -H-E-mK and RABV-TNF $\alpha$ (mem)-H-E-mK respectively. Rows C and Ci show images of virus rescue of the positive control virus, RABV- EBLV-1-eGFP taken at passage one and three respectively. Row D shows fluorescence from the transient transfection of TNF $\alpha$ -GFP.

### 3.2.7.5 Are the 8xHis-tag and E-tag responsible for the apoptotic phenotype associated with cell death?

To investigate whether cell death was being caused by the 8xHis and E-tag located between the 62-71-3 scFv and mCHERRY, these genes were removed. Section 2.23.9 and Figure 3:15 describes the removal of the 8xHis and E-tag and the resulting construct was named RABV-

62scFv. Figure A9 displays the fully assembled RABV-62scFv construct and the PCR products involved in its creation.

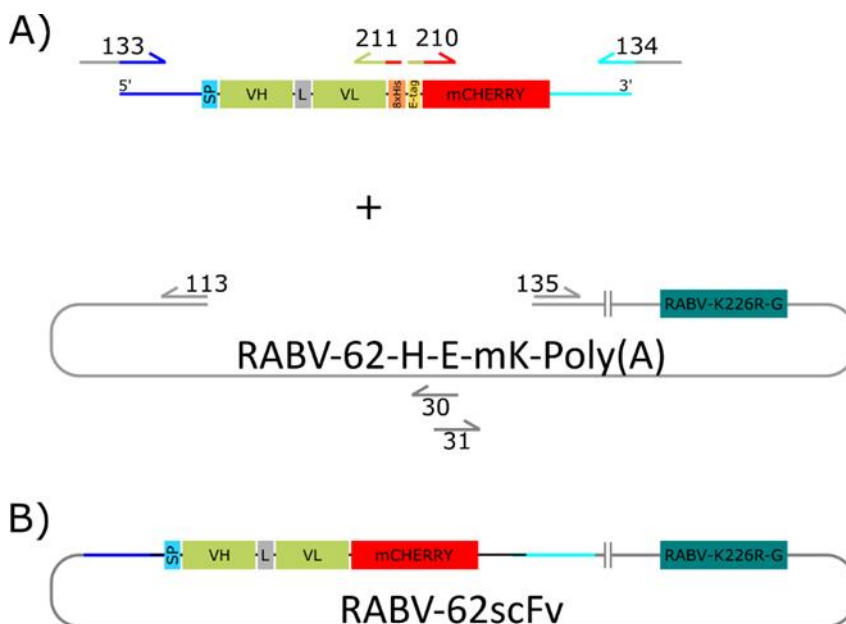


Figure 3:15: Schematic showing the cloning strategy involved in the creation of RABV-62scFv. To remove the 8xHis and E-tag from the 62-71-3 scFv cassette of RABV-62-H-E-mK-Poly(A), the 62-71-3 scFv was amplified in two parts using oligonucleotides 133/211, and 210/134. After the linearisation of RABV-62-H-E-mK-Poly(A) using oligonucleotide primers 113/30, and 135/31 these fragments were then assembled to form construct RABV-62scFv (B). All oligonucleotide primers have been colour coded to indicate homologous regions where dark blue indicates homology to the transcriptional start unit, green to VL region, red to mCHERRY, light blue to the transcriptional stop unit, and grey to the RABV-62-H-E-mK-Poly(A) backbone. The sequences of all oligonucleotide primers shown are listed in Appendix 2.

After confirmation by Sanger sequencing, RABV-62scFv was taken forward for virus rescue. Figure 3:16 shows UV images of RABV-62scFv virus rescue. After transfection of RABV-62scFv into BSR-T7/5 cells, mCHERRY fluorescence was seen throughout cell culture (Figure 3:16A). Furthermore, this fluorescence could be maintained over multiple passages and resulted in the development of fluorescent foci (Figure 3:16B). RABV- EBLV-1-eGFP was used as a positive control for virus rescue and was successfully rescued (Figure 3:16C/D).

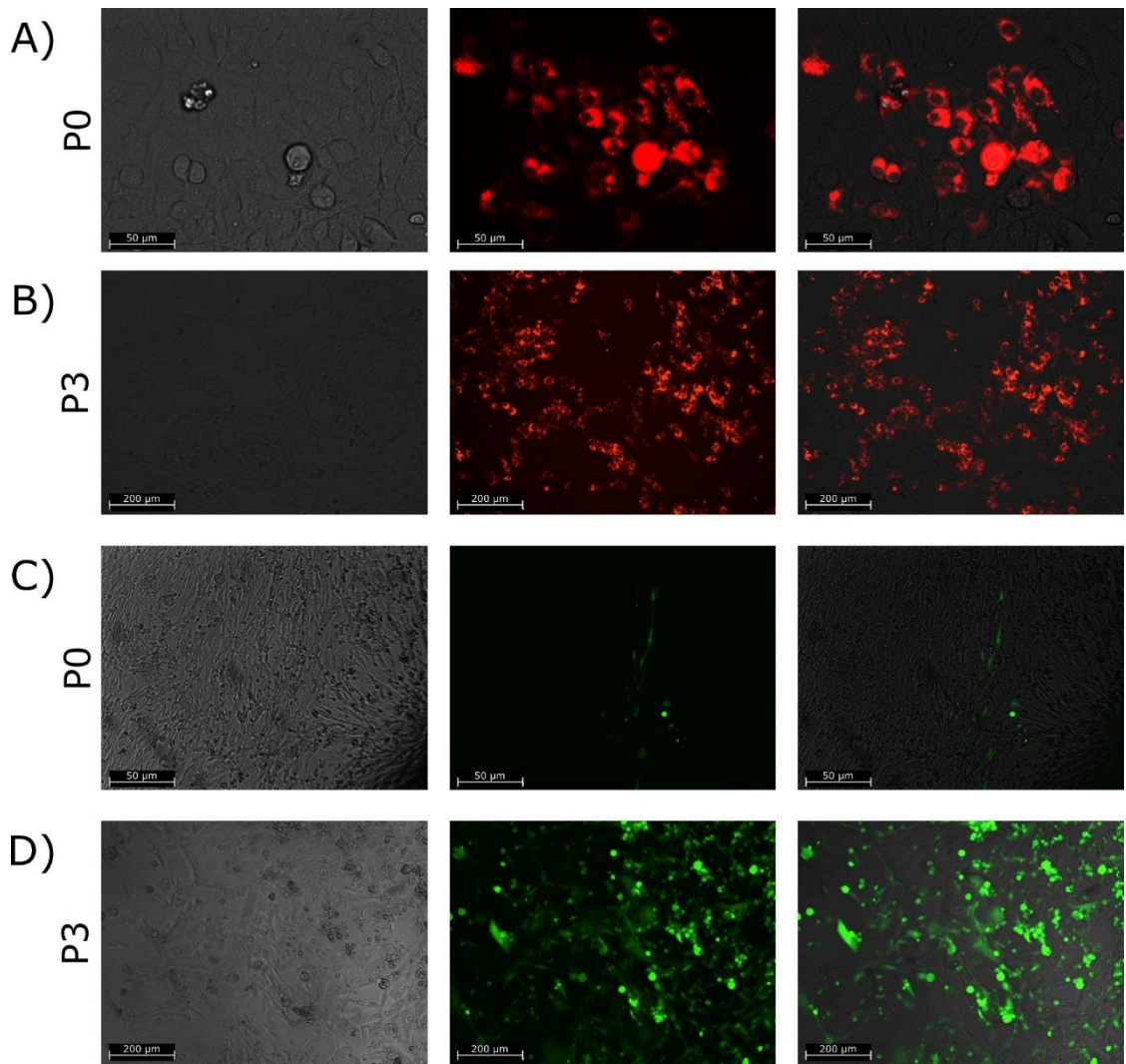


Figure 3:16: UV microscope images of RABV-62scFv and RABV- EBLV-1-eGFP. From left to right, images are brightfield, UV, and a merged image. A, shows images after transfection (passage 0) of RABV-62scFv. B, shows RABV-scFv culture after three passages. C and D show images from the positive control of virus rescue (RABV- EBLV-1-eGFP) at passages 0 and three.

### 3.3 DISCUSSION

62-71-3 scFv was previously expressed in *N. benthamiana* plants using the pTRAK.6 plant expression vector<sup>302,308</sup> and was located within an expression cassette which allowed secretion into the extracellular environment. This cassette incorporated a human heavy chain SP, the 62-71-3 scFv, an 8xHis tag, an E-tag, and a DsRED tag for fluorescent visualisation under UV. To develop a system enabling the expression of the 62-71-3 scFv behind the BBB, this expression cassette was inserted into the genome of an attenuated RABV vector.

Rabies virus rescue has previously been undertaken by transiently transfecting five plasmids simultaneously into BHK-21 cells <sup>79</sup>. These five plasmids encode RABV genes N, P, M, full-length RABV genome, and a source of T7-polymerase (all N/P/M helper plasmids are under T7 promoter control). To optimise this process, various methods of eliciting T7-polymerase expression were investigated. As described in Section 3.2.2, the BSR-T7/5 cell line consistently expressed the highest level of T7-polymerase and so this cell line was used for all future RABV rescues.

For future *in vivo* studies, the presence of 62-71-3 scFv in the extracellular media is vital. The 62-71-3 scFv expression cassette described previously contained an *O. sativa* leader sequence to enable expression <sup>308</sup>. This sequence was later identified as a human heavy-chain SP <sup>331</sup>. Human heavy-chain SPs are commonly used in scFv production in CHO cells, however, to assess whether this SP was effective in promoting secretion from BSR-T7/5 cells after expression from a RABV, two nanoluciferase constructs (pNL1.1 62-NLuc and pNL1.1 sec62-NLuc) were created. As demonstrated in Section 3.2.3, these constructs allowed the quantitative measurement of 62-71-3 scFv secretion from BSR-T7/5 cells. Here, it was shown that the human heavy chain SP preceding the 62-71-3 scFv was effective in promoting secretion of 62-71-3 scFv into the extracellular media of mammalian cell culture.

After the optimisation of T7-polymerase expression in RABV rescue and confirmation of the effectiveness in the human heavy-chain SP in promoting secretion of 62-71-3 scFv, the 62-71-3 scFv cassette was inserted into RABV vaccine strain RABV-cSN. The 62-71-3 scFv cassette was chosen to be inserted upstream of the RABV N gene within the RABV-cSN vector for two reasons. Firstly, due to the transcriptional gradient in RABV replication arising from the disassociation of the polymerase between intergenic regions, genes towards the 3' end are produced in much higher amounts <sup>80</sup>. Therefore, insertion of the 62-71-3 scFv here would enable the highest expression of 62-71-3 scFv. Secondly, while NVEs have not been inserted into the RABV genome upstream of the RABV N gene previously <sup>180</sup>, this is a common location for non-viral element insertion in VSV. Importantly, the insertion of NVEs in this location has been consistently effective in attenuating virus pathogenicity <sup>332-334</sup> and many VSV vectors with non-viral element insertions at the 3' end have been used successfully in *in vivo* models with reduced pathogenicity.

Lastly, RABV gene borders were inserted to flank the 62-71-3 scFv cassette. Gene borders (transcriptional stop and start units) are conserved across the *Mononegavirales* order. In RABV, these non-translated intergenic regions comprise a Poly(A)denylation signal (<sup>A</sup>/<sub>T</sub>G(A)<sub>7</sub>),

an un-transcribed region (C(N)<sub>1-23</sub>), and a transcriptional start sequence for the following gene (AACAC<sub>T</sub><sup>C</sup>/T<sub>T</sub>NCU)<sup>79</sup>. To ensure the production of scFv, the 62-71-3 scFv cassette was flanked by the transcriptional start and stop units from the RABV P gene.

Following the insertion of the 62-71-3 scFv cassette into RABV-cSN, a series of optimisations were performed to allow efficient virus rescue. First, DsRED was replaced with mCHERRY. DsRED is an obligate tetramer both *in vitro* and *in vivo* which poses issues in its use as a fluorescent tag for intracellular components where some reports have described intracellular aggregation of chimeric DsRED constructs<sup>325</sup>. Furthermore, DsRED has a slow maturation half time of ~11 h<sup>326</sup>. In contrast, a derivative of DsRED, mCHERRY, does not require tetramerisation granting higher tolerance to N-terminal fusions, is highly photostable, and has a significantly enhanced maturation time of 15 minutes<sup>335</sup>. Furthermore, mCHERRY has been widely used in RABV studies as a C-terminal fusion protein<sup>85,336,337</sup>. The presence of an effective fluorophore greatly simplifies RABV rescue, where confirmation of successful rescue can be given by visualisation under UV and not by anti-RABV N protein immunocytochemistry. An effective fluorophore labelling the 62-71-3 scFv will also allow the visualisation of 62-71-3 scFv in mouse tissues in future animal studies.

Next, as discussed in Section 3.2.5.3, the G gene of the RABV-cSN vector carrying the 62-71-3 scFv cassette underwent a K226R mutation to encode an arginine residue at position 226 to avoid self-neutralisation<sup>302</sup>. The transcriptional start and stop sequences flanking the 62-71-3 scFv cassette were also replaced to better match those of the positive control virus RABV-EBLV-1-eGFP (Guanghui Wu, APHA, personal communication). These alterations are described in detail in Section 3.2.5. In brief, the RABV N gene Poly(A) tail upstream of the scFv cassette was removed and a Poly(A) tail was incorporated downstream. The resulting construct, RABV-62-H-E-mk-Poly(A), was then taken forward to virus rescue.

Initial rescue attempts of RABV-62-H-E-mk-Poly(A) were unsuccessful while the positive control for virus rescue (RABV- EBLV-1-eGFP) was consistently rescued. Specifically, while cellular fluorescence was present three to four days after transfection at passage 0, cell death soon occurred and after passage no fluorescent foci formed. It was previously thought that any mCHERRY fluorescence under UV was indicative of successful virus rescue, as mCHERRY should only be produced at the advent of both the transcription and translation of all RABV-62-H-E-mk-Poly(A) proteins. However, as mCHERRY fluorescence was frequently observed at three to four days post-transfection (in the absence of successful virus rescue), successful virus rescue

could only be confirmed by the presence of fluorescent foci after multiple passages. The cause of the apoptotic phenotype and cell death was then investigated.

As discussed previously (Section 3.2.3), BSR-T7/5 cells were used for virus rescue due to their innate ability to express high levels of T7-polymerase. To assess whether this cell death was specific to BSR-T7/5 cells, virus rescue was attempted in multiple different cell lines including: BHK-21, BSR-T7/5, CHO, HEK-293T, and N2a cells. As indicated, virus rescue was uniformly unsuccessful in all cell types investigated. Cells would frequently become fluorescent three to four days after initial transfection (passage 0), but no fluorescent foci could be observed after multiple passages. This confirmed that the cell death which followed fluorescent cells in Figure 3:9 was not confined to the BSR-T7/5 cell line.

As discussed previously, the mutation of the RABV G gene at site 226 from a K to an R was performed to avoid binding of 62-71-3 scFv. However, previous research has shown that this mutation does not abolish binding, only diminishes it <sup>302</sup>. Therefore, it was hypothesised that cell death may be arising from the residual binding of 62-71-3 scFv to the mutated RABV G protein. As has been discussed previously, the RABV G protein undergoes reversible structural transitions (native, active, and fusion-inactive states) during synthesis and transport through the ER-Golgi apparatus due to differing pH environments <sup>57,58,338</sup>. Due to significant antigenic differences between the native state (present on mature virus particles) and the active and fusion-inactive states, it is likely that 62-71-3 binds only RABV G protein in the native state (although this has not been conclusively demonstrated). This presented the possibility that 62-71-3 scFv was binding RABV G protein trimers on the cell surface. Furthermore, previous research has shown that antibody binding to cell surface RABV G protein is able to redirect the intracellular destination of RABV G protein in an Fc-independent manner <sup>339</sup>.

During its production and characterisation, 62-71-3 was shown to effectively bind and neutralise many phylogroup I Lyssaviruses including CVS-11, EBLV-2 (strain RV1787) and ABLV (*Australian Bat Lyssavirus*, strain RV634). However, in this study no binding was observed to DUVV (Duvnshage Virus, strains SA06 and RV131), EBLV-1 (strains RV9 and RV20), and LBV Lyssavirus (Lagos Bat Virus), a phylogroup II Lyssavirus <sup>302</sup>. This is a common phenomenon with phylogroup I Lyssavirus-neutralising antibodies where cross-neutralisation does not often occur between phylogroups I and II and rarely occurs between phylogroup III <sup>311,340</sup>. To assess whether 62-71-3 scFv was binding to the mutated RABV G protein, RABV G gene was exchanged for IKOV G gene, the glycoprotein of a highly divergent phylogroup III Lyssavirus <sup>341</sup>. To confirm the inability of 62-71-3 scFv to bind and neutralise IKOV, binding was assessed by

cytochemistry and neutralisation assessed by FAVN assay. Here, as shown in Figure 3:11, incubation of *N. benthamiana* produced 62-71-3 scFv DsRED on IKOV infected cells revealed 62-71-3 scFv was unable to bind IKOV G protein on infected cells. In contrast, 62-71-3 scFv DsRED was able to effectively bind G protein present on the surface of CVS-11 infected cells. These results were reflected in the FAVN assay, where 62-71-3 scFv DsRED was able to neutralise CVS-11 at all concentrations tested, but a neutralising titre for IKOV could not be defined. After the successful exchange of the RABV G gene for the IKOV G gene the successful construct, RABV-62-H-E-mCH-IKOV, was taken forward to virus rescue within BSR-T7/5 cells. However, virus rescue remained unsuccessful with cells progressing to cell death after the observation of fluorescence under UV. This suggests that residual binding to the RABV G protein was not the underlying cause of unsuccessful virus rescue.

Whilst this demonstrated that cell death was unlikely to be due to residual binding of RABV G protein, it was conceivable that the apoptotic phenotype may be arising from non-specific binding of 62-71-3 scFv to intracellular or cell-membrane bound components. To investigate this, two RABV constructs were designed. In one construct, 62-71-3 scFv was replaced with a secreted TNF $\alpha$  (RABV-TNF $\alpha$ -H-E-mK) and in the other was replaced with a non-secreted TNF $\alpha$  (RABV-TNF $\alpha$ (mem)-H-E-mK). Both of these constructs were based upon a previous publication by Faber et al.<sup>253</sup> where secreted and non-secreted versions of TNF $\alpha$  were designed for insertion into an attenuated RABV expression vector. Furthermore, Faber et al demonstrated that TNF $\alpha$ -expressing RABV only displayed an approximate 15% and 30% decrease in cell viability for non-secreted and secreted TNF $\alpha$  expressing variants respectively in NA cells. After successful cloning, RABV-TNF $\alpha$ -H-E-mK and RABV-TNF $\alpha$ (mem)-H-E-mK were taken forward to virus rescue. All virus rescues were unsuccessful with cells quickly progressing to cell death. Furthermore, fluorescent foci could not be visualised after multiple passages. These observations suggest that unsuccessful virus rescue was not due to the non-specific binding of 62-71-3 scFv to membrane-bound or intracellular components.

Up to this point, cell death arising during virus rescue had been investigated thoroughly and was found not to be due to residual self-neutralisation, cell line type, or non-specific binding of scFv. It was then decided to investigate whether apoptotic phenotype development was due to another aspect of the 62-71-3 scFv cassette. Thus, the region encoding the 8xHis and E-tag in between the 62-71-3 scFv and mCHERRY was removed. The 8xHis and E-tags were remnants from the expression of 62-71-3 scFv within *N. benthamiana*<sup>302,308</sup> where the 8xHis was included to enable purification of 62-71-3 scFv from whole plant leaf extract using a nickel-

affinity column and the E-tag included for detection in western blot labelling (8xHis could also be used for this application). However, in the context of expression within RABV neither of these tags were deemed necessary. Furthermore, if needed, the mCHERRY tag could be used for detection in western blot labelling. Deletion of these tags resulted in the construct RABV-62scFv which was taken forward to virus rescue. As shown in Figure 3:16, RABV-62scFv virus rescue was successful with no apoptotic phenotype present and the development of fluorescent foci after multiple passages. This suggests that the cell death present in previous RABV rescue was due to the presence of the 8xHis and E-tag region between the 62-71-3 scFv and mCHERRY fluorophore. Interestingly, both 8xHis and E-tag have been used within mammalian expression systems previously without detrimental effect <sup>342,343</sup> (although E-tags are most commonly used in *E. coli* expression systems <sup>344-347</sup>). However, no data exist from previous publications on the expression of His/E-tagged scFv proteins within mammalian cell culture by either RABV or VSV. With regards to whether apoptotic phenotype arose from the presence of the 8xHis or E-tag, the presence of hydrophobic amino acids (61%) encoding the E-tag may have caused intracellular aggregation and cell death <sup>348-350</sup>. However, due to both time constraints and the difficulty associated with virus rescue, no viral constructs were designed (i.e lacking either the 8xHis or E-tag) to investigate this.

## Chapter 4 - IN VITRO VIRUS CHARACTERISATION

### 4.1 INTRODUCTION

Since 2001, various studies have investigated the addition of ORFs containing non-viral elements within the RABV genome (reviewed by Smith et al, 2019 <sup>180</sup>). Mutations of the RABV backbone have also been investigated in an effort to define attenuating mutations for vaccine development and understand pathogenesis <sup>351</sup>. In these studies, the effects of genetic mutation or non-viral element insertion have been measured using various techniques including growth curve analysis, cellular toxicity, and cell-to-cell viral dissemination. By measuring the effect of RABV mutation or non-viral element insertion, it is possible to evaluate the subsequent impact on viral pathogenicity. For example, previous research has suggested that many (but not all <sup>230</sup>) pathogenic RABVs replicate less efficiently *in vitro* (resulting in a lower titre in viral growth curve analysis) but also disseminate faster in *in vitro* experiments (larger foci in cell-to-cell viral dissemination assays) compared to their apathogenic alternatives <sup>228,229</sup>. These characteristics may be related to the mechanisms employed by RABV to evade the host immune response. As seen for attenuated viruses, early activation of the immune response is associated with rapid clearance of infection in the periphery <sup>125</sup>. To avoid this, pathogenic RABVs express significantly lower levels of G protein, create fewer progeny RABVs, and possess multiple mechanisms of immune evasion <sup>197,352-354</sup>. However, the few progeny RABVs that are produced possess an enhanced ability to infect, replicate, and disseminate cell-to-cell compared to attenuated viruses <sup>228</sup>. In the present study, multiple *in vitro* experiments were performed to characterise RABV-62scFv and to assess aspects including cytotoxicity, viral growth kinetics, and cell-to-cell dissemination.

While analysis of RABV-62scFv replication, dissemination, and infection will give potential insight to *in vivo* pathogenicity, 62-71-3 scFv expression and secretion is imperative for the clearance of symptomatic rabies from the CNS. Therefore, in addition to viral replication, cell-to-cell dissemination and infectivity, 62-71-3 scFv expression was also assessed. First, the presence of vp62-71-3 scFv was investigated in RABV-62scFv infected cell culture supernatant by western immunoblotting. Heat-inactivated supernatant from RABV-62scFv infected cell culture was used to assess the ability of vp62-71-3 to bind RABV G protein by staining an acetone-fixed RABV-infected cell culture. To assess the ability of vp62-71-3 to neutralise infectious virus, heat-inactivated supernatant from RABV-62scFv infected cell culture was used to neutralise live virus in a FAVN test. Together, these experiments were used to confirm the

expression of 62-71-3 scFv from RABV-62scFv-infected cell culture and to give an indication of RABV-62scFv pathogenicity when administered *in vivo* in a mouse model.

## 4.2 RESULTS

### 4.2.1 Assessing Cellular Toxicity of RABV-62scFv infection

As discussed in Section 3.2.7, cells infected with previous versions of RABV-62scFv which still contained both an 8xHis and E-tag displayed a granular fluorescence phenotype, which was associated with cell death. However, as shown previously (Figure 3:16, Section 3.2.7.5), cells infected with RABV-62scFv retained this granular fluorescence but remained alive.

To assess any significant detrimental effect of RABV-62scFv infection, N2a cells (ATCC® CCL-131™) were infected with either RABV-62scFv or RABV-cSN and cell viability was assessed by measuring the mitochondrial reduction of an MTS tetrazolium compound into a coloured formazan product. N2a cells are of mouse neuroblastoma origin and were selected as the cell line most closely resembling the murine CNS. Furthermore, N2a cells have been shown to be highly sensitive to RABV infection<sup>355,356</sup> and NA cells (a subclone) have been used in previous studies when assessing toxicity of novel recombinant RABV constructs<sup>253</sup>. The results, as shown in Figure 4:1, revealed no significant differences between cells infected with either RABV-cSN or RABV-62scFv.

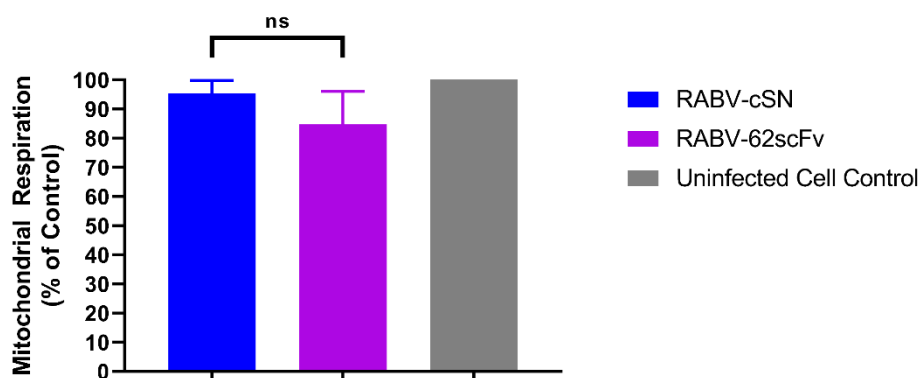


Figure 4:1: Assessing the effect of RABV-62scFv infection on mitochondrial respiration. An MTS assay was performed on N2a cells to assess the effect of RABV-62scFv and RABV-cSN infection on N2a cell viability. After the assay had been performed and absorbance read, values were compared against the uninfected cell control to obtain value for mitochondrial respiration.

Experiment was performed in triplicate to  $n=3$ . Results were analysed using the Brown-Forsythe and Welch ANOVA Test ( $F^* 5.299$ ) where  $ns P > 0.05$ .

#### 4.2.2 Multi-Step Virus Growth Curves

Growth kinetics of RABV-62scFv was assessed by multi-step growth curves in both BHK-21 and N2a cells alongside three other viruses: RABV-cSN, RBV-mCH, and RABV-K226R-mCH (Figure 4:2). RABV-cSN is the backbone vector in which all mutations and insertions were introduced (see Section 3.2.5). RABV-mCH was generated through the insertion of mCHERRY upstream of the N gene in RABV-cSN. RABV-K226R-mCH was based upon RABV-mCH, where the glycoprotein gene was subject to a K226R mutation.

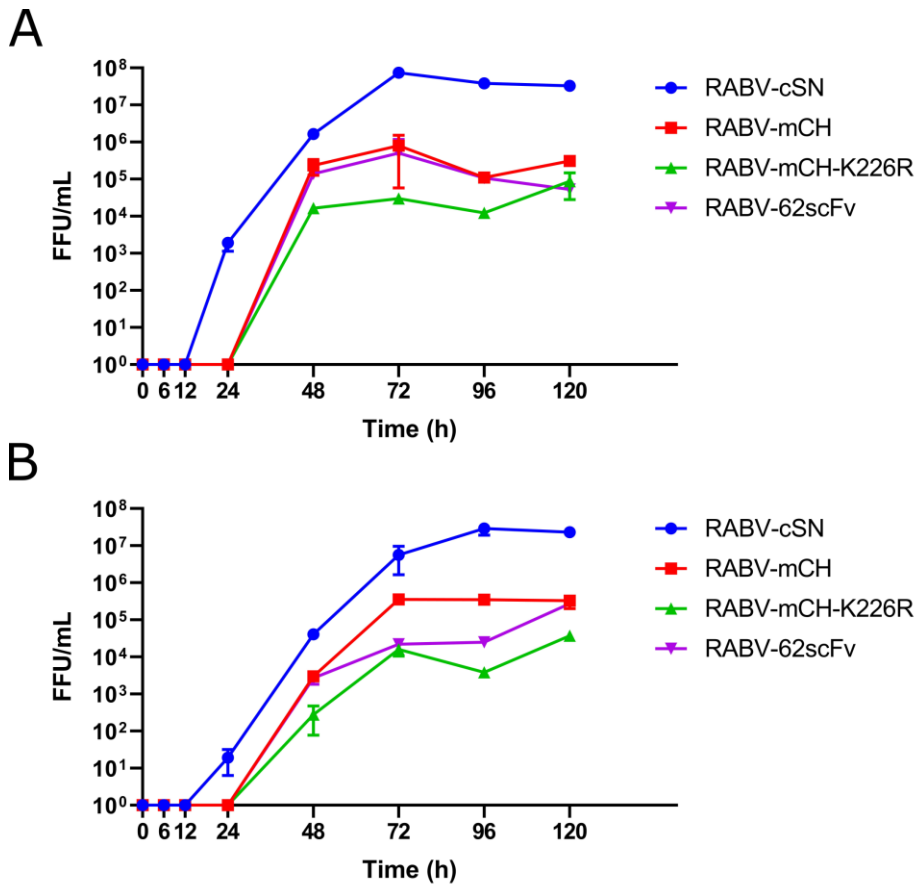


Figure 4:2: Growth kinetics of RABV-cSN, RABV-mCH, RABV-mCH-K226R, and RABV-62scFv. Growth kinetics were assessed by multi-step growth curves in both BHK-21 (A) and N2a cells (B). After 1 h infection of either a BHK-21 or N2a cell monolayer at 0.01 MOI, supernatant was taken at 0, 6, 12, 24, 48, 72, 96, and 120 h before FFU/mL measured by virus titration. Experiment was performed in triplicate to  $n=3$ .

N2a or BHK-21 cells were infected with either RABV-cSN, RABV-mCH, RABV-mCH-K226R, or RABV-62scFv and samples taken at various timepoints. After the final sample collection, virus samples were titrated (Section 2.12.2) and growth curve analysis undertaken (Section 2.14).

As shown in Figure 4:2, in both BHK-21 and N2a cells, RABV-cSN was detectable after 24 h incubation. In BHK-21 cells (Figure 4:2A), RABV-cSN plateaued from 72 h, while in N2a cells (Figure 4:2B) this was from 96 h. In both N2a and BHK-21 cells, RABV-mCH, RABV-mCH-K226R, and RABV-62scFv were present from the 48 h timepoint. In BHK-21 cells (Figure 4:2A), all three virus titres plateaued at 48 h, while in N2a cells (Figure 4:2B) plateauing was only observed from 72 h.

The mean titre of each virus, upon plateauing, was evaluated for significant differences to compare virus growth between RABV-cSN, RABV-mCH, RABV-mCH-K226R, and RABV-62scFv. As shown in Table 4:1, statistical analysis showed significant differences between the post-plateau titres of RABV-cSN and RABV-mCH, and RABV-mCH and RABV-mCH-K226R. This was consistent between growth curves performed in both BHK-21 and N2a cells. In BHK-21 cells however, a significant difference in the post-plateau titres of RABV-mCH-K226R and RABV-62scFv was also observed. This significant difference was not present in growth curves performed in N2A cells.

|                | BHK-21                 |                          | N2A                    |                          |
|----------------|------------------------|--------------------------|------------------------|--------------------------|
| Virus          | Plateau titre (FFU/mL) | Statistical Significance | Plateau titre (FFU/mL) | Statistical Significance |
| RABV-cSN       | 4.83E+07               | ***<br>**<br>*           | 2.62E+07               | **<br>***<br>ns          |
| RABV-mCH       | 2.51E+05               |                          | 3.45E+05               |                          |
| RABV-mCH-K226R | 2.93E+04               |                          | 1.89E+04               |                          |
| RABV-62scFv    | 8.76E+05               |                          | 3.05E+04               |                          |

Table 4:1: Statistical differences between mean titres of RABV-cSN, RABV-mCH, RABV-mCH-K226R, and RABV-62scFv after plateauing in growth curves. In BHK-21 cells, RABV-cSN plateaued at 72 h, while RABV-mCH, RABV-mCH-K226R, and RABV-62scFv plateaued at 48 h. In N2a cells, RABV-cSN plateaued at 96 h, while RABV-mCH, RABV-mCH-K226R, and RABV-62scFv plateaued at 72 h. To investigate the effect of mCHERRY insertion, RABV G gene K226R mutation, and 62-71-3 scFv insertion, RABV-cSN was compared to RABV-mCH, RABV-mCH to RABV-mCH-K226R, and RABV-mCH-K226R to RABV-62scFv respectively. A Brown-Forsythe and

Welch ANOVA test was performed where \*\*\*  $P \leq 0.001$ , \*\*  $P \leq 0.01$ , \*  $P \leq 0.05$ , and ns  $P > 0.05$ . F\* test values for BHK-21 and N2A statistical tests were 25.21 and 74.97, respectively.

Together, Figure 4:2 and Table 4:1 illustrate the impact of mCHERRY insertion, RABV G gene K226R mutation, and 62-71-3 scFv insertion on RABV growth kinetics. As shown, the insertion of mCHERRY prior to the N gene adversely affected RABV growth, as did the K226R mutation of the RABV G gene. Interestingly, the addition of the 62-71-3 scFv into the same ORF as mCHERRY only affected growth kinetics when growth curves were performed in BHK-21 cells and not N2a cells. Furthermore, when comparing virus titres between N2a and BHK-21 cells, a significant difference was only observed in titre of RABV-62scFv ( $p > 0.05$ , Brown-Forsythe and Welch ANOVA test).

#### 4.2.3 In vitro virus dissemination assay

To investigate the impact of both the RABV G gene K226R mutation and the insertion of the 62-71-3 scFv-mCHERRY cassette on the ability of RABV-62scFv to disseminate from cell to cell, an *in vitro* virus dissemination assay was developed. Described in Section 2.21, this involved the infection of an N2a cell monolayer with a low titre (0.001 MOI) of virus and the addition of an agarose overlay. Virus dissemination was determined by counting the average number of infected cells within each fluorescent focus. A percentage increase value was generated for each timepoint (48, 72, 96, and 120 h) relative to 24 h.

The kinetics of cell-to-cell viral dissemination of RABV-62scFv was compared against five viruses: RABV-cSN, RABV-mCH, RABV-mCH-K226R, RABV-CVS-mCHERRY, and CVS-11 allowing the effect of both the insertion of mCHERRY, 62-71-3 scFv, and the RABV G gene K226R mutation on cell-to-cell viral dissemination to be analysed. Furthermore, as cell-to-cell viral dissemination is indicative of virus pathogenicity, a comparison against known pathogenic viruses RABV-cSN, RABV-CVS-mCH, and CVS-11 was included to provide an indication of the potential pathogenicity of RABV-62scFv.

Figure 4:3 shows the results of this experiment performed in N2a cells. These cells were selected due to their murine neuronal origin and, therefore, results should be more applicable to neuronal infection in a mouse model. This experiment was attempted in BHK-21 cells as the virus may have become BHK-21 adapted after multiple passages during virus rescue. However, despite multiple attempts under different conditions (agarose content, media serum concentration, and incubator temperature/CO<sub>2</sub> concentration) BHK-21 cells could not survive overlay and extensive cell death occurred after 48 h.

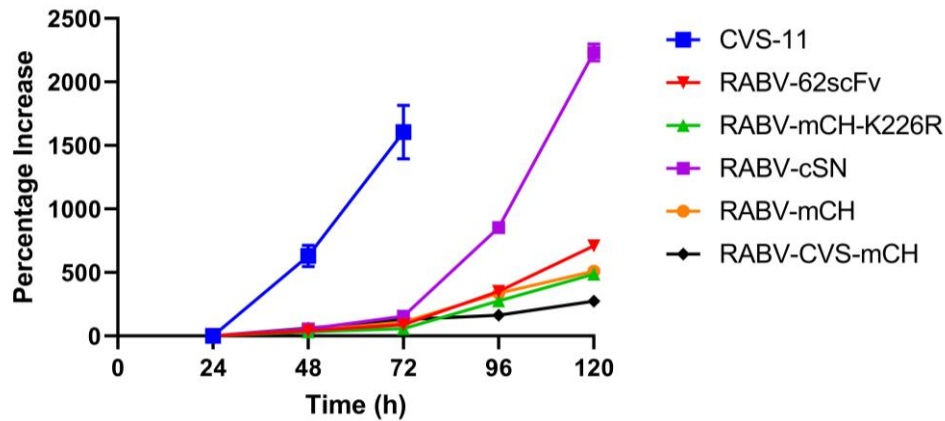


Figure 4:3: Viral dissemination assay investigating the differences between the cellular dissemination of RABV-cSN, RABV-mCH, RABV-mCH-K226R, RABV-62scFv, CVS-11, and RABV-CVS-mCH over 120 h. 100 foci were observed per timepoint per virus and cells per foci counted. A percentage increase value was then generated for each timepoint relative to 24 h. Readings of CVS-11 was terminated after 72 h due to exponential increase. This experiment was repeated in triplicate to n=3 and the mean percentage increase values displayed.

As shown in Figure 4:3, pathogenic viruses RABV-cSN, RABV-CVS-mCH, and CVS-11 exhibited vastly different cell-to-cell viral dissemination patterns. While both RABV-cSN and CVS-11 displayed notably larger foci at earlier timepoints, RABV-CVS-mCH did not disseminate from cell-to-cell easily and foci size remained small throughout all timepoints. RABV-mCH, RABV-mCH-K226R, and RABV-62scFv foci size, similar to RABV-CVS-mCH, remained small throughout all timepoints assessed.

Interestingly, the phenotype of the foci generated by infection differed between the RABV viruses. The foci generated by RABV-cSN and CVS-11 infection were consistently concise and, as demonstrated by the data in Figure 4:3, foci size grew quickly. This contrasts with the foci generated by RABV-mCH, RABV-mCH-K226R, RABV-62scFv, and RABV-CVS-mCH infection. These viruses did not form concise foci and it became increasingly difficult to distinguish individual foci throughout later timepoints. Figure 4:4 shows representative images of foci formed by RABV-cSN (A/B), RABV-62scFv (C/D), and RABV-CVS-mCH (E/F) infection. As shown, RABV-cSN formed markedly larger foci than RABV-CVS-mCHERRY at equivalent time points.

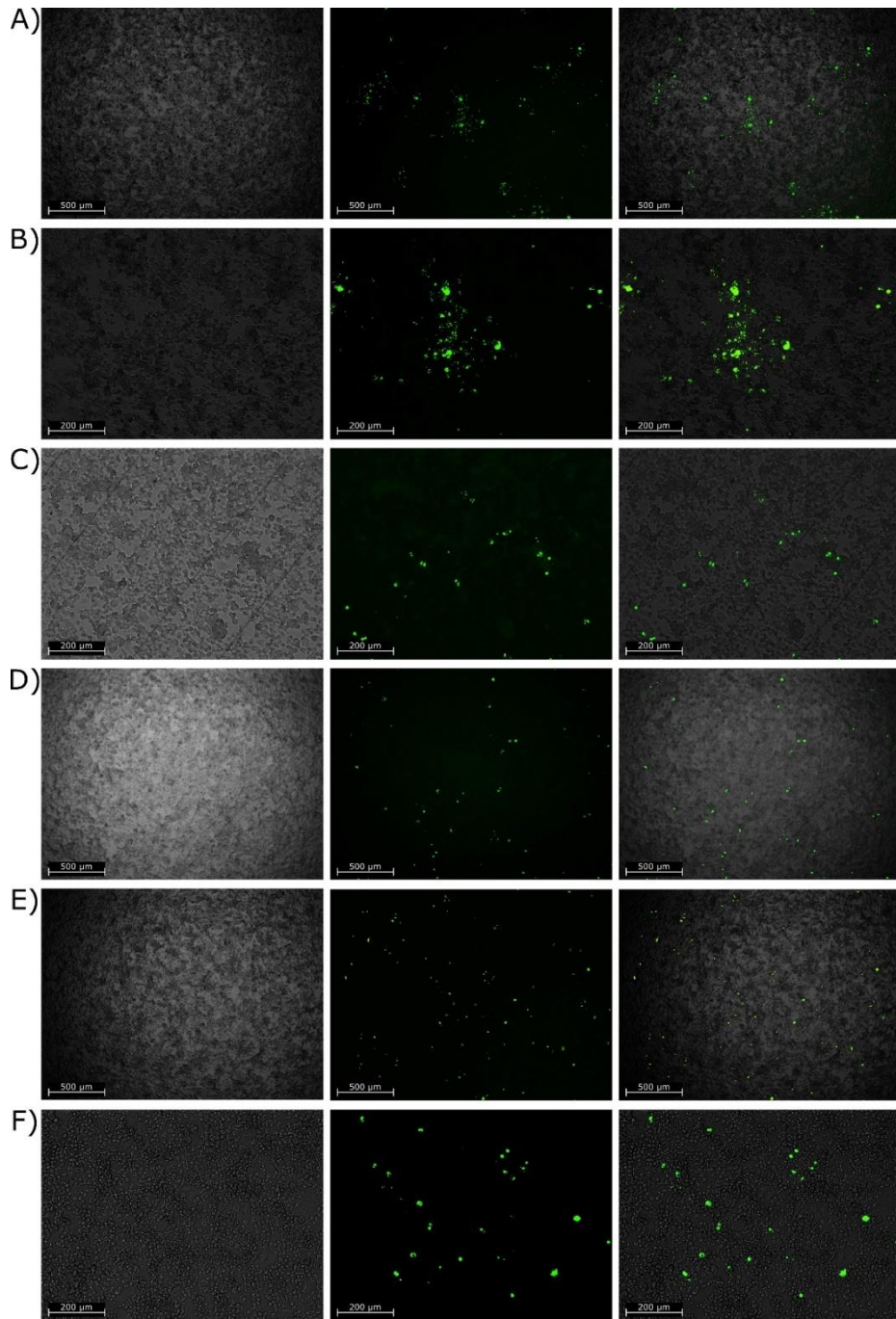


Figure 4:4: Representative images of foci generated in the cellular dissemination assay by either RABV-cSN, RABV-62scFv, or RABV-CVS-mCHERRY infection. A and B show images of foci generated by RABV-cSN infection (A at 10x, B at 4x magnification). C and D show images of foci generated by RABV-62scFv infection at similar magnifications, respectively. E and F show images of foci generated by RABV-CVS-mCH infection at similar magnifications, respectively. Images are displayed as brightfield, FITC fluorescence under UV, and a merged image. All cells have been stained for RABV N protein and visualised under UV. All images taken at 96 h post infection.

The phenotypic differences in foci generated by RABV-cSN and CVS-11 infection compared to cSN-mCH/mCH-K226R/62scFv suggested that the insertion of an mCHERRY-containing ORF upstream of the RABV N gene severely impacted the ability of RABV to disseminate from cell-to-cell. This conclusion was reflected in the observed differences in foci size between mCHERRY and non-mCHERRY containing viruses. The RABV G gene K226R mutation and addition of the 62-71-3 scFv into the mCHERRY ORF had no significant effect on cell-to-cell dissemination.

#### 4.2.4 Production of 62-71-3 scFv over time

To assess the production of 62-71-3 scFv over time after BHK-21 cell culture infection, an ELISA was performed. Detailed in Section 2.15, this experiment was based upon the Platelia® Rabies II Kit (BioRad) with major experimental modifications involving the use of a Rat anti-mCHERRY (5F8, chromotek) and HRP Goat anti-rat antibody (405405, BioLegend). Together, these antibodies allowed the detection of 62-71-3 scFv by the binding of the C-terminal mCHERRY fluorophore. After infecting BHK-21 cells with RABV-62scFv and RABV-mCH-K226R, supernatant was removed at 0, 4, 8, 24, 72, and 96 h. After storage at -80°C an ELISA was performed, and the results detailed in Figure 4:5.

As shown, 62-71-3 scFv remained undetectable in the supernatant of infected cells until after 24 h, where exponential expansion was seen through the 72 and 96 h time points. To demonstrate that OD450 signal was due to 62-71-3 scFv binding, RABV-mCH-K226R was included as a control as this virus lacks the 62-71-3 scFv gene.

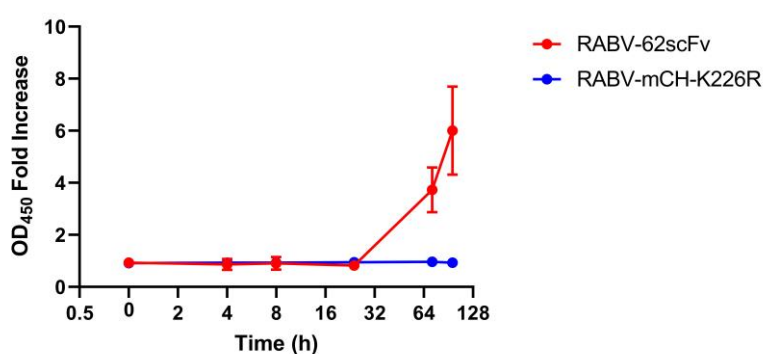


Figure 4:5: vp62-71-3 scFv production over time after BHK-21 cell culture monolayer infection with RABV-62scFv. Supernatant from RABV-mCH-K226R has been included as a control and results are shown as fold-increase of the negative media-only control. Where possible, error bars representing the standard error of the mean have been shown.

#### 4.2.5 Identification of vp62-71-3 scFv using protein immunoblotting.

As discussed previously, excretion of 62-71-3 scFv into the extracellular media of infected cells is the intended mechanism for neutralisation of RABV within the CNS of infected animals. To confirm the presence of 62-71-3 scFv in the extracellular media of RABV-62scFv infected cells (termed vp62-71-3 scFv), cell culture supernatant was removed and inactivated using BPL. Appropriate controls were included such as inactivated media from cells infected with RABV-cSN, RABV-mCH and RABV-K226R. Inactivated cell culture media was run on an SDS-PAGE gel before being analysed by immunoblotting by an anti-mCHERRY antibody (see Section 2.4.6).

As shown in Figure 4:6, immunoreactive bands were generated in immunoblots with the supernatant of RABV-mCH (A), RABV-mCH-K226R (B), and RABV-62scFv (C) infected cell culture, but not in the supernatant of cells infected with RABV-cSN (D). The band present in A and B, representing supernatant from RABV-mCH and RABV-mCH-K226R infected cells, is approximately 27 kDa. This is the expected size for monomeric mCHERRY (26.7 kDa). The large bands present in C (denoted by arrows), representing supernatant from RABV-62scFv infected cells, was much larger (56 kDa) than mCHERRY bands present in Lanes A and B, suggesting that mCHERRY was being detected as part of the 62-71-3 scFv polyprotein (estimated size 56 kDa). However, the additional presence of the 27 kDa band in this figure suggests that mCHERRY is being post-translationally cleaved from the 62-71-3 scFv polyprotein to form monomeric mCHERRY.

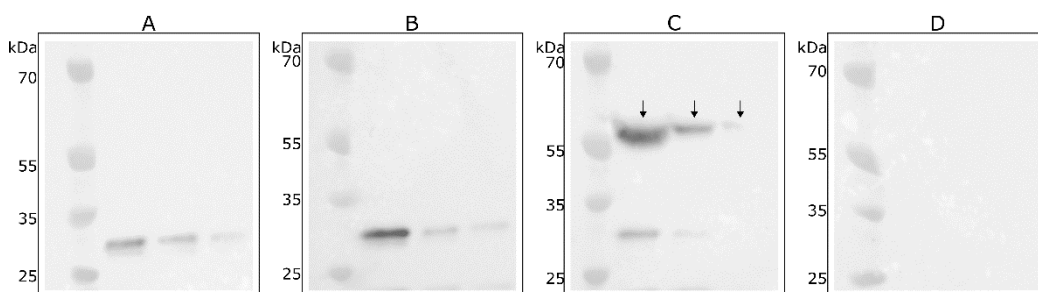


Figure 4:6: Western Blot of supernatant from infected cell culture. Cells were infected with either RABV-mCH (A), RABV-mCH-K226R (B), RABV-62scFv (C), or RABV-cSN (D). After infection, any virus present in the supernatant was inactivated by BPL treatment. Supernatant was titrated in doubling dilutions and all dilutions ran on an SDS-PAGE gel before transfer to a nitrocellulose membrane. To visualise bands, nitrocellulose membranes were stained with an anti-mCHERRY (6G6, chromotek) antibody. stained with an anti-mCHERRY (6G6, chromotek) antibody.

#### 4.2.6 Investigating the ability of vp62-71-3 to bind RABV G protein using immunocytochemistry

The ability of the heat-inactivated supernatant of RABV-62scFv-infected cells to bind RABV G protein was assessed to confirm whether the mCHERRY-containing protein reported in Section 4.2.5 was vp62-71-3 scFv.

Here, BHK-21 cells were infected with either CVS-11 (Figure 4:7) or RV437 (Figure 4:8). RV437, a pathogenic RABV strain isolated from a Raccoon-dog (*Nyctereutes procyonoides*) (GenBank Accession number: KF154997), was included in this experiment due to its planned use in subsequent *in vivo* mouse challenge models (Chapter 5). RABV -infected cells were fixed in acetone and stained for RABV N protein (FITC Anti-Rabies Monoclonal Globulin, Fujirebio). Supernatant from RABV-62scFv-infected cells was then incubated on fixed cells after heat-inactivation. Images were taken under UV light and the results shown in Figure 4:7 and Figure 4:8 below.

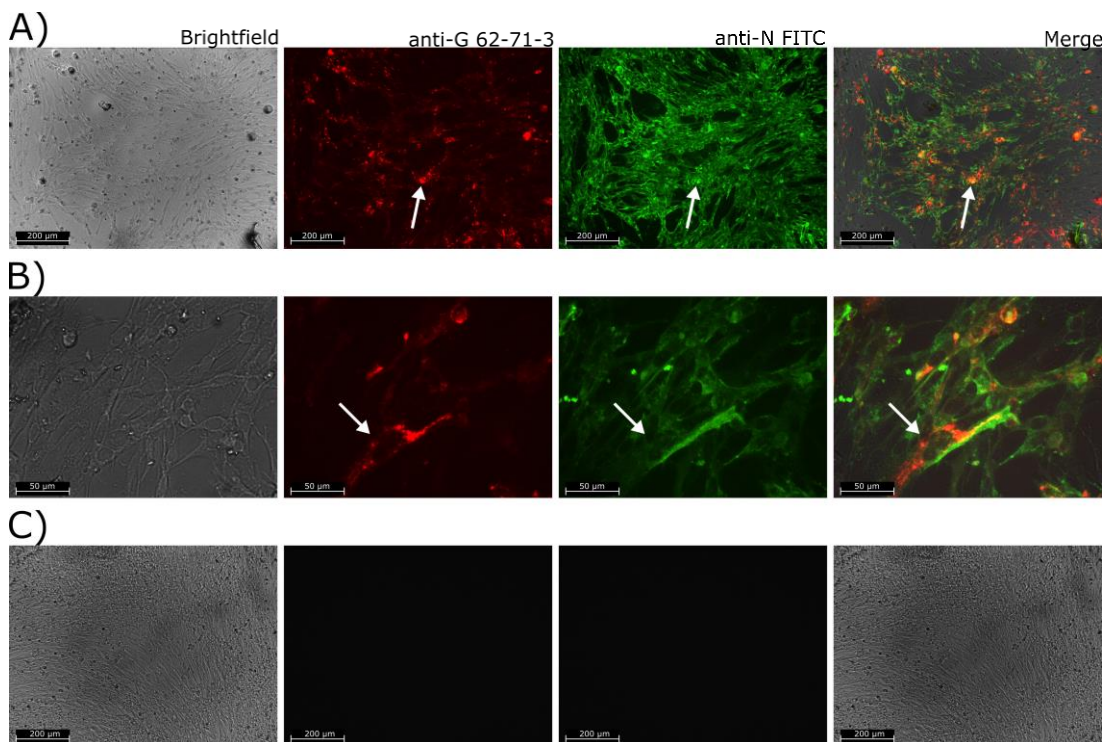


Figure 4:7: CVS-11-infected BHK-21 cells fixed and stained for RABV N and G protein. Row A and B show BHK-21 cell culture infected with CVS-11 RABV and fixed with 80% acetone. A is shown in 100x magnification, while B is shown in 400x magnification. Each column has been labelled to indicate whether cells are shown in brightfield, stained with anti-RABV N protein FITC antibody (Fujirebio), stained for RABV G protein using heat-inactivated supernatant from RABV-62scFv infected cell culture, or are displayed as a merge of all three images. C, shows

uninfected BHK-21 cells at 100x magnification which have been stained and illuminated for RABV G and N protein as previously described. White arrows indicate cells of interest.

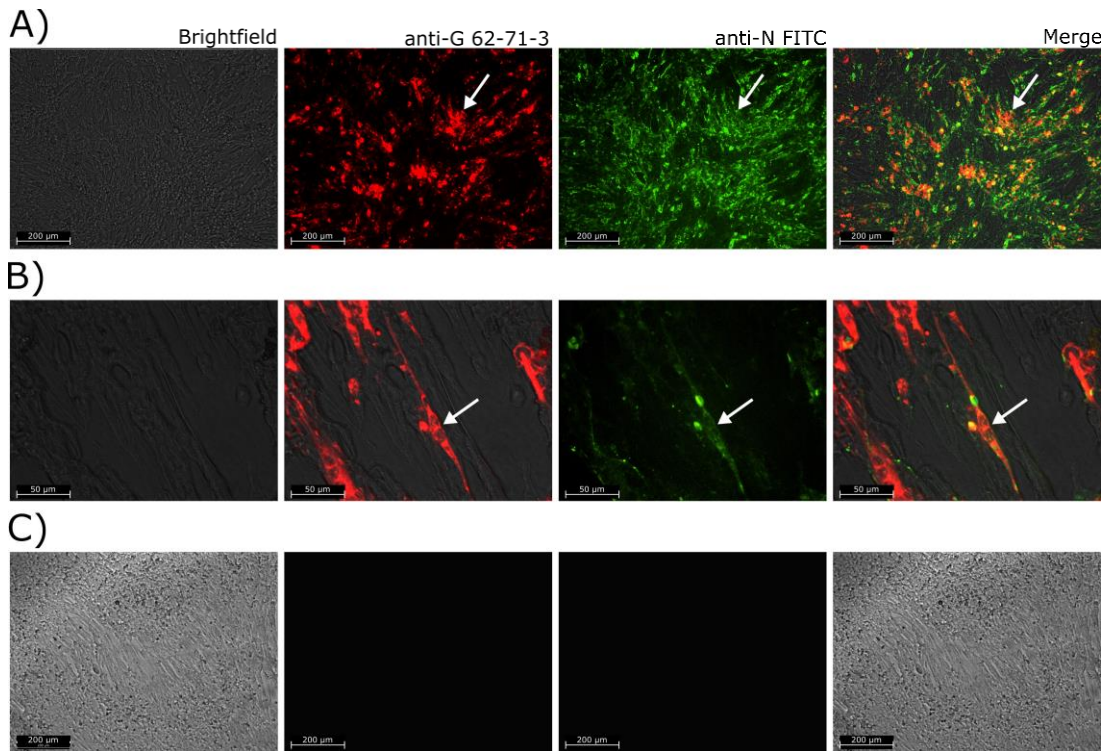


Figure 4:8: RV437 infected BHK-21 cells fixed and stained for RABV N and G protein. Here, row A (100x magnification) and B (400x magnification) show BHK-21 cell culture infected with RV437 RABV and fixed with 80% acetone. Each column has been labelled to indicate whether cells are shown in brightfield, stained with anti-RABV N protein FITC antibody (Fujirebio), stained for RABV G protein using heat-inactivated supernatant from RABV-62scFv infected cell culture, or are displayed as a merge of all three images. C, shows uninfected BHK-21 cells which have been stained and illuminated for RABV G and N protein as previously described (Section 2.16). White arrows indicate cells of interest

As shown by the co-localisation of the green RABV N protein staining and the red RV437/CVS-11 G protein staining, vp62-71-3 scFv from RABV-62scFv-infected cell supernatant was effective in binding fixed cells infected with CVS-11 and RV437.

#### 4.2.7 Assessing Neutralisation Activity of vp62-71-3 scFv using the Fluorescent Antibody Virus Neutralisation (FAVN) Test

While the ability of vp62-71-3 scFv to bind RABV G protein has been shown in Section 4.2.6, binding does not necessarily lead to viral neutralisation<sup>357,358</sup>. A modified FAVN test was performed to assess whether binding will lead to viral neutralisation. Here, as described in

Section 2.17, vp62-71-3 scFv was serially diluted and used to neutralise 100 TCID<sub>50</sub> of either RABV-CVS-11 or RV437. To ensure that 62-71-3 scFv was the cause of any neutralisation observed, supernatant from cells infected with RABV-mCH-K226R (which lacks the 62-71-3 scFv gene) was used to assess neutralisation of CVS-11 and RV437.

As shown in Table 4:2, vp62-71-3 scFv was able to neutralise both CVS-11 and RV437 to equal extents, while supernatant from cells infected with RABV-mCH-K226R was unable to neutralise either.

| Challenge Virus | Sera assessed for neutralisation | Neutralising/non-neutralising | Reciprocal titre / IU/mL |
|-----------------|----------------------------------|-------------------------------|--------------------------|
| <b>CVS-11</b>   | vp62-71-3                        | Neutralising                  | 12.14 / 0.39             |
| <b>CVS-11</b>   | RABV-mCH-K226R supernatant sera  | Non-Neutralising              | n/a                      |
| <b>CVS-11</b>   | Cell Culture Media Control       | Non-Neutralising              | n/a                      |
| <b>RV437</b>    | vp62-71-3                        | Neutralising                  | 13.09 / n/a              |
| <b>RV437</b>    | RABV-mCH-K226R supernatant sera  | Non-Neutralising              | n/a                      |
| <b>RV437</b>    | Cell Culture Media Control       | Non-Neutralising              | n/a                      |

Table 4:2: Results of a FAVN test investigating the ability of vp62-71-3 scFv to neutralise both CVS-11 RABV and RV437. As a control, supernatant from RABV-mCH-K226R infected cells was used to assess CVS-11 neutralisation. Results are displayed as whether vp62-71-3 scFv was neutralising or non-neutralising and is accompanied by an IU/mL or reciprocal titre value. Experiment was repeated three times and reciprocal titres averaged.

Traditionally, a FAVN test results rely on the generation of an international unit per mL (IU/mL) value. This is because the FAVN test is used widely in the assessment of the level of serum RABV neutralising antibody present in vaccinated individuals. As discussed previously, if this value is above 0.5 IU/mL then an individual is regarded to have sero-converted, which implies a protective immunity against RABV infection. However, IU/mL can only be generated if the FAVN is performed against CVS-11, if another virus is used (in this case RV437) a reciprocal titre value is generated.

#### 4.2.8 Ability of RABV-62scFv to cross an *in vitro* Blood-Brain Barrier Model

A potent localised neutralising antibody-mediated immune response is required for the clearance of RABV from the CNS during symptomatic infection. If administered peripherally, RABV-62scFv must be able to cross the BBB in order to produce RABV-neutralising vp62-71-3 scFv within the CNS to enable RABV clearance. An *in-vitro* BBB model was used to investigate the ability of RABV-62scFv to cross the BBB. This involved the addition of RABV-62scFv to the hCMEC/D3 monolayer with media samples taken from both the apical and basolateral side at various timepoints (Section 2.22).

Following the successful seeding of hCMEC/d3 cells on to the semi-permeable transwell membrane, LY was used to assess the integrity in the hCMEC/D3 monolayer permeability over time. As shown in Figure 4:9, the LY apparent permeability coefficient (LY  $P_{app}$ ) decreased significantly after 120 h, indicating sufficient formation of TJs between hCMEC/D3 cells. Furthermore, across all time points, LY recovery was approximately 75%. Therefore, all further BBB model experiments were performed 120 h after seeding.

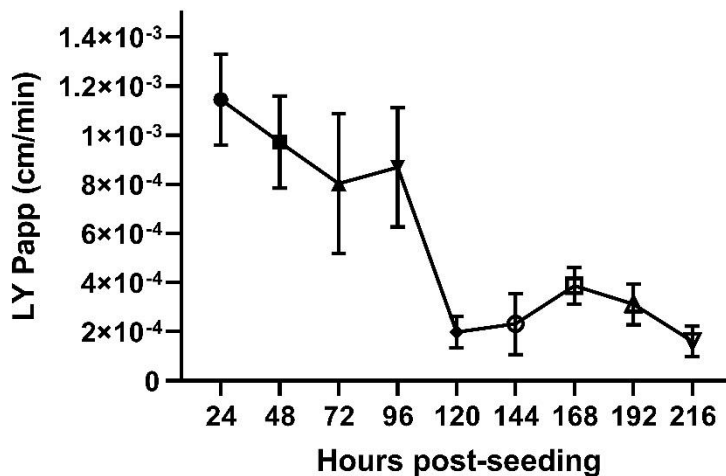


Figure 4:9: Kinetics of hCMEC/D3 BBB model formation over time. At each 24 h time point after cell seeding, an apparent permeability ( $P_{app}$ ) value was calculated.

The ability of RABV-62scFv to cross the hCMEC/D3 monolayer was assessed as described in Section 2.22.3. 72 h after infection of the hCMEC/D3 monolayer with RABV-62scFv, media was removed for RNA extraction and RT-PCR (using oligonucleotide primers described in Appendix 2) to investigate whether infection of the hCMEC/D3 monolayer resulted in virus production on the basolateral side of the transwell.

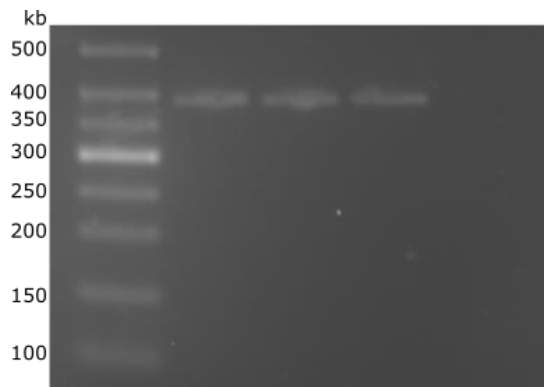


Figure 4:10: Assessment of the presence of RABV-62scFv to cross a hCMEC/D3 BBB. 72 h after hCMEC/D3 monolayer infection with RABV-62scFv, RNA was extracted from both the apical and basolateral compartments and amplified for RABV-specific sequences. The image shows the molecular weight ladder used, followed by apical and basolateral samples taken from wells infected with RABV-62scFv, respectively. The latter two lanes display apical and basolateral samples taken from wells treated with heat-inactivated RABV-scFv.

As shown in Figure 4:10, 72 h after infection RT-PCR detected RABV-scFv RNA in both the apical and basolateral compartments of the BBB model. In contrast, hCMEC/D3 monolayers that were treated with heat-inactivated RABV-62scFv revealed presence of RNA in the apical compartment only. To support the hypothesis that RABV RNA presence in the basolateral compartment was due to the infection of the hCMEC/D3 monolayer, images were taken of the hCMEC/D3 monolayer after RABV-62scFv infection under UV illumination. As shown in Figure 4:11, red cell-localised fluorescence was observed, which is indicative of RABV-62scFv cell infection.

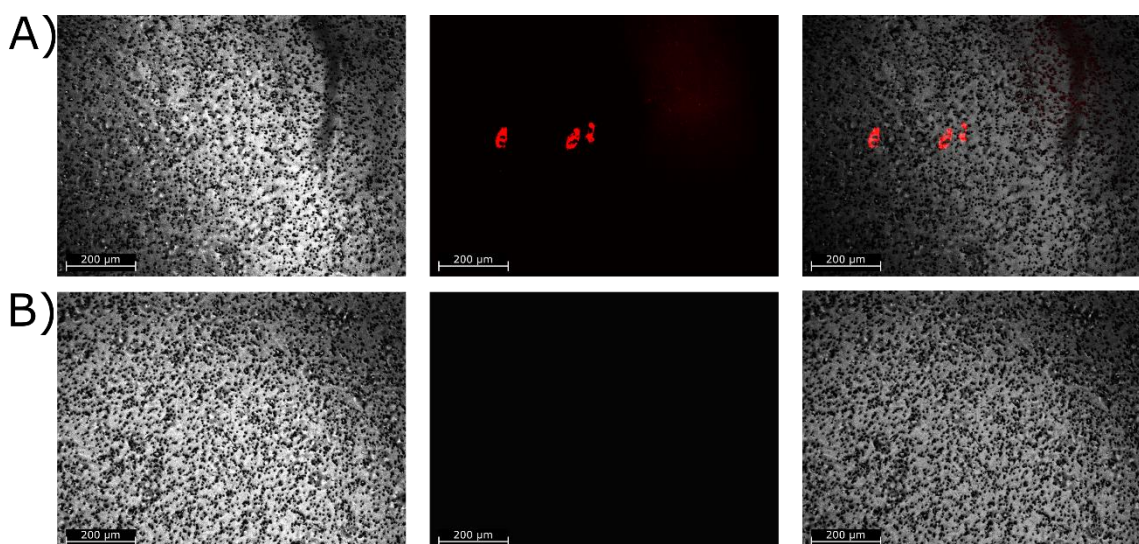


Figure 4:11: Images taken of a hCMEC/D3 blood-brain barrier monolayer to illustrate cellular infection with RABV-62scFv. Row A shows brightfield, UV illumination, and overlay images of hCMEC/D3 cells treated with live RABV-62scFv, while row B shows images taken from hCMEC/D3 cells treated with heat-inactivated RABV-62scFv.

### 4.3 DISCUSSION

This chapter has described the in vitro characterisation of RABV-62scFv using methods including cell viability assays, multi-step growth curves, and cell-to-cell viral dissemination. Further, various RABV-62scFv aspects have also been investigated such as the immunoblotting of vp62-71-scFv, assessing its ability to bind RABV G protein binding, and RABV neutralisation properties. The ability of RABV-62scFv to cross an in-vitro BBB was also assessed.

Cell viability upon infection was assessed due to concerns regarding the phenotype of granular fluorescence present upon RABV-62scFv infection. This phenotype had previously been observed in cells expressing 62-71-3 scFv containing the 8xHis and E-tag (Section 3.2.7). In these cells, the granular fluorescence phenotype had been consistently accompanied by cell death. While visual inspection indicated that infection with RABV-62scFv lacking affinity tags did not appear to result in excessive cell death, the granular fluorescence phenotype remained and consequently MTS assays were employed to measure cell viability. Data from cell viability assays confirmed that cell viability was unaffected by infection with RABV-62scFv and further studies with the virus were initiated. The granular phenotype observed upon RABV-62scFv infection was, therefore, attributed to the production of the scFv-mCHERRY polyprotein within cells. If the fluorescent granular phenotype was indeed not a precursor to cell death, which was occurring due to the presence of 8xHis and E-tag within the scFv cassette, then the

fluorescent granules may be the scFv-mCHERRY polyprotein being shuttled from the ER/Golgi pathway to the cell surface possibly due to the presence of the SP. While unfortunately this experiment could not be performed, this hypothesis of intracellular transport of scFv-mCHERRY to cell surface could be confirmed by images taken over a time course by using confocal microscopy.

The growth kinetics of RABV-62scFv was also assessed by multi-step growth curve analysis. In contrast to single-step growth curves where cells are infected by a high titre of virus, multi-step growth curves are more representative of virus growth *in vivo*. Furthermore, where single-step growth curves only show the growth kinetics in a single infection-replication event, multi-step growth curves also consider cell-to-cell viral dissemination. Multi-step viral growth curves were performed in both N2a and BHK-21 cells and results shown in Figure 4:2. As RABV-62scFv has undergone multiple mutations and insertions, a series of viruses were constructed to assess the impact of each insertion or mutation: comparison between RABV-cSN, RABV-mCH, RABV-mCH-K226R, and RABV-62scFv revealed the impact of mCHERRY ORF insertion upstream of the RABV N gene, the K226R mutation, and the addition of the 62-71-3 scFv into the existing mCHERRY ORF. Interestingly, there was only a significant difference in growth kinetics upon the insertion of 62-71-3 scFv into the existing mCHERRY ORF in BHK-21 cells, not N2a cells. This suggests, as N2a cells are of murine neuronal origin, that while the K226R mutation may be of importance in BHK-21 cell culture-based culture there may be no impact in neuronal infection in *in vivo* murine models. However, it is important to note that this hypothesis has been drawn from *in vitro* results, and further *in vivo* investigation is needed to confirm the impact of the K226R RABV G gene mutation on RABV replication within in the mouse model. The significant impact on growth kinetics associated with the introduction of an ORF upstream of the RABV N gene was thought to be due to the transcriptional gradient elicited by the presence of intergenic regions present in the RABV genome<sup>80</sup>. The insertion of an ORF (and therefore an additional IGR) upstream of the RABV N gene meant that there was another location for polymerase dissociation to occur, and subsequently all downstream proteins were produced at lower levels. The impact of the RABV G gene K226R mutation on RABV growth kinetics is an observation reported in many other studies investigating attenuating G protein mutations<sup>228,232</sup>. Where assessed, impacted growth kinetics has been attributed to increased cellular apoptosis, lower G protein expression, or decreased cell-to-cell viral dissemination. While data from the cellular toxicity study indicated that impacted growth kinetics was not due to increased apoptosis, lower G protein expression was not assessed. The cell-to-cell viral dissemination of RABV-62scFv, however, was assessed and will be discussed

below. An alternative explanation for impacted viral growth kinetics may be attributed to the need for optimal interactions between RABV G and M proteins during viral replication <sup>229</sup>. However, as only the cytoplasmic domain of G protein interacts with the M protein in viral budding, the mutation of aa 226 is unlikely to affect this interaction.

The growth kinetics of novel recombinant RABVs reveal more insights into their functionality, replication, and pathogenicity. Multiple studies have linked replication rate to RABV pathogenicity, where the lower replication rate of pathogenic viruses may act to prevent the induction of cell death which may hinder retrograde axonal travel of RABV particles and activation of the immune system <sup>229,237,240,318,359</sup>. Therefore, these findings provided an indication that RABV-62scFv would display pathogenic properties if administered *in vivo*.

Plaque assays are a common method of titrating cytopathic viruses such as Rift Valley fever virus, severe acute respiratory syndrome coronavirus 2 (SARS-COV-2), and VSV <sup>360-362</sup>.

Furthermore, in some cases, the size of these plaques has also been used to give an insight into virus attenuation <sup>363</sup>. RABV is not a lytic virus and, therefore, a traditional plaque assay cannot be performed. However, as described in Section 2.21, through the use of N2a cells, an agarose-DMEM overlay, and an anti-RABV N protein fluorescent antibody, it is possible to measure the cell-to-cell dissemination of RABV. Other workers have performed similar RABV dissemination assays and in multiple studies cell-to-cell viral dissemination has been consistently correlated to *in vivo* pathogenicity upon intracranial infection <sup>36,228,229,364</sup>. As demonstrated in Figure 4:3, RABV-62scFv did not exhibit rapid cell-to-cell dissemination and foci rarely became larger than five cells. This contrasted with the results obtained with CVS-11 and RABV-cSN, which are both pathogenic RABV strains upon intracranial *in vivo* infection, the former also pathogenic upon peripheral infection. As shown, these viruses quickly displayed an exponential increase in the number of cells per foci. Interestingly, RABV-CVS-mCH displayed a similar pattern of cell dissemination to RABV-62scFv but not to CVS-11 or RABV-cSN despite both being pathogenic *in vivo* following intracranial administration <sup>365</sup>. One explanation for this is based in the construction of RABV-CVS-mCH. Unlike other genetically engineered RABVs previously described, RABV-CVS-mCH contains an additional NVE-containing ORF before the N-gene. Other NVEs in RABV have predominantly been inserted between the G and L genes <sup>180</sup>. Mirroring the theory to explain the reduced growth kinetics seen by RABV-62scFv in Figure 4:2, the presence of an additional ORF upstream of the RABV N gene may have affected the transcriptional gradient and resulted in the reduced production of downstream proteins. Further evidence in support of this explanation is provided by the difference in the foci sizes of

RABV-cSN and RABV-mCH, where the only difference is the insertion of mCHERRY as the 3' proximal transcription cassette. Traditionally, the reduced dissemination of RABV-62scFv would suggest that RABV-62scFv is apathogenic upon *in vivo* infection, however, the reduced dissemination of RABV-CVS-mCH indicates that reduced cell-to-cell viral dissemination is not necessarily indicative of attenuation.

The proposed mechanism of action for RABV-62scFv involves the release of vp62-71-3 scFv into the extracellular media of infected cells. Multiple experiments were performed to assess the presence and functionality of this vp62-71-3 scFv. First, after infecting BHK-21 cell culture with RABV-62scFv, supernatant was BPL inactivated and underwent analysis by western blot using an anti-mCHERRY antibody. As shown in Figure 4:6, bands correlating to vp62-71-3 scFv were identified in supernatant samples from RABV-62scFv infected cells. Interestingly, an additional band was present in the supernatant from cells infected with RABV-mCH, RABV-mCH-K226R, and RABV-62scFv. The size of this band correlated with monomeric mCHERRY. While the mCHERRY present in RABV-mCH and RABV-mCH-K226R was not accompanied by a secretory signal, its presence in cell culture supernatant may be due to the release of mCHERRY upon cell death. The additional presence of this band in the supernatant from RABV-62scFv infected cells suggested that, in addition to full-length vp62-71-3 scFv-mCHERRY, monomeric mCHERRY was also being produced upon RABV-62scFv cellular infection. How monomeric mCHERRY was being produced was unknown, however, there were two potential mechanisms. First, mCHERRY may have been cleaved from the vp62-71-3 scFv polyprotein by host proteases. This hypothesis was supported by observations from previous studies investigating 62-71-3 scFv expression<sup>308</sup>. In this study, 62-71-3 scFv was linked to DsRED instead of mCHERRY, and a 8xHis and E-tag encoded between the scFv and DsRED. Here, western blot analysis of 62-71-3 scFv produced in *N. benthamiana* plants revealed the presence of two bands, a 56 kDa (expected size) and a smaller 30 kDa band described simply as a "degradation product"<sup>308</sup>. By using the predicted molecular weights of these aa sequences, in addition to scFv fragments and DsRED, the smaller fragment seen on western blot can be attributed to a cleavage in the (Gly<sub>4</sub>Ser)<sub>3</sub> linker between the variable heavy and light chains of the scFv<sup>308</sup>. However, the size of the smaller band present consistently in supernatants from RABV-mCH, RABV-mCH-K226R, and RABV-62scFv in Figure 4:6 indicates that this monomeric mCHERRY presence was not a product of (Gly<sub>4</sub>Ser)<sub>3</sub> linker cleavage but possibly a cleavage of the region between mCHERRY and the scFv. One alternative hypothesis for monomeric mCHERRY production after RABV-62scFv infection was the translation of mCHERRY from an in-frame start codon. Here, similar to the underlying mechanism of P protein isoform production

<sup>25</sup>, mCHERRY may be translated alone from the in-frame start codon present at the beginning of the mCHERRY gene in the 62-71-3 scFv cassette. Cellular resources are potentially being unnecessarily spent on the production of monomeric mCHERRY if monomeric mCHERRY arises through translation from an in-frame start codon. In downstream studies, this may be avoided by the removal of mCHERRY from the 62-71-3 scFv cassette as it was not needed for neutralisation of RABV.

After establishing the growth and virus dissemination kinetics of RABV-62scFv, experiments were undertaken to investigate the production of vp62-71-3 scFv into the supernatant of cells infected with RABV-62scFv. Simultaneously, the supernatant of infected cells was assessed by ELISA for the presence of an mCHERRY-conjugated protein able to bind RABV G protein, and how the expression of this protein changed over time. Firstly, by comparing the supernatants of RABV-62scFv and RABV-mCH-K226R infected cells, it was shown that only the supernatant of RABV-62scFv infected cells was able to produce an mCHERRY-labelled RABV G protein binding protein into the extracellular media (presumably vp62-71-3 scFv). By performing a single-step growth curve and removing supernatant at various intervals, it was also shown that the expression of vp62-71-3 scFv remained undetectable until the 72 h timepoint where vp62-71-3 scFv levels increased exponentially. These data correlate well with the RABV-62scFv kinetic growth data shown in Figure 4:2. In both of these graphs, RABV-62scFv (or vp62-71-3 scFv) levels increased exponentially after 24 h. However, while virus levels plateaued after 48 h in BHK-21 cells, 62-71-3 scFv mCHERRY levels continued to exponentially increase. This indicated that, within the context of *in vitro* cell culture infection, vp62-71-3 scFv was more stable than RABV-62scFv. While RABV-62scFv levels reached an equilibrium of viral degradation and viral production after 48 h, vp62-71-3 scFv was able to persist for longer and did not reach an equivalent plateau in the timepoints assessed.

Cytochemistry on RABV-infected cells was performed to further assess the ability of vp62-71-3 scFv to bind RABV G, especially those of viruses used in downstream experiments. Here, a monolayer of BHK-21 cells was infected with either CVS-11 or RV437. These viruses were selected as 62-71-3 had previously been shown to neutralise infective CVS-11 virus<sup>302</sup> and RV437 was the virus strain of choice for downstream *in vivo* mouse challenge studies (Chapter 5). After infection of a cell monolayer, cells were fixed and stained with both an anti RABV N protein FITC conjugated antibody (Fujirebio) and supernatant from RABV-62scFv infected cells. As shown in Figure 4:7 and Table 4:2, red fluorescence from 62-71-3 scFv-mCHERRY binding localised consistently with green RABV N protein staining on both CVS-11 and RV437 infected

cells, indicating that vp62-71-3 was able to bind the glycoproteins of both CVS-11 and RV437 present on infected cells.

As discussed previously, antibody binding does not necessarily lead to virus neutralisation<sup>357,358</sup>. To assess the ability of vp62-71-3 scFv to neutralise infective virus, modified FAVN (mFAVN) tests were performed against CVS-11 and RV437. As shown in Table 4:2, vp62-71-3 scFv was effectively able to neutralise both CVS-11 and RV437 to similar extents. In this experiment, the titre value (reciprocal or international units per mL) generated was relative to the concentration of 62-71-3 scFv present in the infected cell culture supernatant. Therefore, this value relies on the period of time that the cell culture was infected by RABV-62scFv as more scFv will be produced over time during infection. Infecting BHK-21 cells in a 6-well plate vs a T-25 flask, for example, produced very different titre results (data not shown). From this experiment, two conclusions could be drawn. Firstly, vp62-71-3 scFv was able to neutralise both CVS-11 and RV437. Secondly, as demonstrated by the similar reciprocal titre values, vp62-71-3 scFv shows similar neutralisation properties for both CVS-11 and RV437.

Lastly, as RABV-62scFv had undergone multiple non-viral element insertions and RABV G gene mutations, the ability of RABV-62scFv to cross an *in vitro* BBB model was assessed. This was important, as intravenous (iv) administration of RABV-62scFv in subsequent *in vivo* experiments was a possibility. Here, as RABV clearance from the CNS is reliant on the production of antibody within the CNS<sup>105,125</sup>, to effectively treat CNS RABV infection iv delivered RABV-62scFv would need to be able to cross the BBB to infect cells within the CNS and enable antibody production.

In previous work, 62-71-3 scFv conjugated to a short 29 aa RABV G peptide (which allowed receptor binding) was shown to be effectively transported across a hCMEC/D3 BBB model<sup>366</sup>. Therefore, functional RABV virions were expected to be able to infect the hCMEC/D3 monolayer. Here, hCMEC/D3 cells were used to form a cell monolayer on a permeable transwell membrane to imitate a BBB. After showing that hCMEC/D3 cells took 120 h to form a confluent monolayer with appropriate integrity, RABV-62scFv was used to infect this hCMEC/D3 monolayer and media samples taken from the basolateral side after 72 h. These media samples were assessed for RABV RNA by RT-PCR. In this experiment, due to the exclusionary nature of the hCMEC/D3 monolayer, heat-inactivated RABV-62scFv (included as a control) would not be able to infect the hCMEC/D3 monolayer and no viral RNA would be present in the basolateral compartment of the transwell insert. In contrast, live RABV-62scFv would be allowed to infect the hCMEC/D3 monolayer and produce RABV-62scFv virions into

the basolateral compartment. As shown in Figure 4:10, RABV-62scFv RNA was present in samples taken from both apical and basolateral compartments of hCMEC/D3 monolayers infected with RABV-62scFv, while RABV-62scFv RNA was only detected in the apical compartment of hCMEC/D3 monolayers treated with heat-inactivated RABV-62scFv. The presence of RABV-62scFv RNA on the basolateral side of the hCMEC/D3 monolayer shows that RABV-62scFv retained its ability to infect BBB cells (an observation supported by Figure 4:11) and indicates RABV-62scFv would be an effective treatment if delivered iv.

## Chapter 5 - IN VIVO ASSESSMENT OF RABV-62scFv

### 5.1 INTRODUCTION

Historically, the first successful LAVV was used in the eradication of smallpox in 1980<sup>180</sup>. Since then, various LAVVs have been developed to combat diseases such as poliovirus, yellow fever virus, rinderpest virus, measles virus, and mumps virus<sup>222</sup>. Rabies LAVVs are currently only used for the protection of animals in mass-vaccination campaigns. However, there is an emerging body of research focussing on the attenuation of rabies viruses by one of three methods. RABV genes may be subject to mutation<sup>351</sup>, genes within RABV may be rearranged, duplicated, or exchanged for genes from other RABV/Lyssaviruses<sup>238,239</sup>, and NVEs may be inserted within the RABV backbone<sup>180</sup>.

Regardless of the method used, attenuation is achieved by the same mechanism. As explored within Smith et al<sup>180</sup> and in Section 1.6.1.1.2, viral attenuation is primarily achieved by the activation of the immune system, an event which does not occur (or occurs too late) in pathogenic RABV infection. With the activation of the immune system, infection of mouse models with attenuated RABVs have revealed activation of DC, T, and GC B cells and the influx of these cells into the CNS through a permeabilised BBB<sup>180</sup>. In particular, within the context of survival after RABV infection, studies using JHD<sup>-/-</sup> and Rag-2<sup>-/-</sup> ko mice, which lack B cells, or both B and T cells respectively, have demonstrated the importance of B cells. For example, the infection of JHD<sup>-/-</sup> and Rag-2<sup>-/-</sup> with attenuated RABVs reveals that without B cells mice quickly succumb to infection. JHD<sup>-/-</sup> mice are able to promote long-term survival after RABV infection before developing symptoms while Rag-2<sup>-/-</sup> mice quickly succumb<sup>105,126</sup>. This indicates the importance of RABV neutralising antibodies in survival after RABV infection. In particular, it is the passage of antibody-producing B cells, rather than the passive leakage of antibody across the BBB that is necessary for clearance of RABV from the CNS<sup>105</sup>.

Considering the necessity of BBB permeabilisation and recruitment of antibody-producing B cells to survival after RABV infection, various studies have investigated the use of highly immunogenic rabies LAVVs in the prevention against RABV mortality when given as a post-exposure treatment. These studies followed on from research demonstrating the ability of LAVVs to effectively and quickly activate the immune system, generating a neutralising anti-RABV antibody response and permeabilising the BBB<sup>180</sup>. Here, LAVVs such as either TriGAS (also known as SPBAANGAS-GAS-GAS)<sup>238</sup>, SRV9<sup>312,313</sup>, or a GM-CSF expressing LBNSE rabies

virus vector<sup>314</sup> have been assessed in their ability to prevent mortality when given up to six days post-lethal challenge where they were effective in a minimum of 20% of mice. These studies indicate that LAVVs represent a potential avenue for the post-clinical onset treatment of rabies. However, it is important to note that these LAVVs were only administered prior to the onset of signs, with diminishing effectiveness as the number of days after lethal challenge increased, so further research may be needed before they are used for true post-onset treatment.

Taking into consideration the need for RABV-neutralising antibody production within the CNS for RABV survival, one study has been able to successfully cure symptomatic RABV in mice using a combination of two monoclonal antibodies (RVC20 and RVC58) delivered by ICV microinfusion pump<sup>310</sup>. By not waiting for the host immune system to generate antibodies of its own, this method of delivering monoclonal antibodies directly into the brain of infected mice allowed an antibody mediated anti-RABV action to begin immediately. The presence of this delay between treatment administration and host-immune system based anti-RABV action may be a key reason for why current LAVV-based treatment options are ineffective at true post-symptom onset treatment and why LAVVs display diminishing effectiveness the longer after lethal challenge they are given<sup>238,312-314</sup>. The delay between treatment administration and anti-RABV action (three to seven days<sup>249-251,253,255-260,265</sup> for serum virus neutralising antibodies (VNA) to appear after ic or im infection) may allow RABV infection to cause too much damage within the infected host.

In summary, there are two currently experimental avenues for RABV treatment; LAVVs and ICV infusion. However, both of these avenues are accompanied by shortcomings. LAVVs are an effective single-dose treatment used frequently in the oral vaccination of wildlife. LAVVs have also consistently been shown to elicit high VNA titres, permeabilisation of the BBB, and recruitment of immune cells into the CNS<sup>180</sup>. However, the lag-period that exists between LAVV infection and VNA production/BBB permeabilisation<sup>94</sup> is a potential problem for post-symptom onset treatment. In contrast, the ICV administration of monoclonal antibodies directly into the brain does not have this lag-period but has been associated with severe procedure-related risks in *in vivo* studies (with three animals dying during ICV infusion) and no demonstration of BBB permeabilisation was performed *in vivo*. In addition, while effective, ICV infusion is an intensive procedure requiring large volumes of RABV-neutralising antibody (a single injection of 20 mg/kg per antibody, in addition to ICV infusion of two mg/kg/day per antibody for 20 days).

The expression of a RABV-neutralising scFv by an attenuated virus vector is suited to combat many of these issues. Firstly, expressing a scFv using a viral vector removes the requirement of the host-immune system to mount an antibody response before RABV clearance can begin. Instead, RABV-neutralising protein production begins upon cellular infection. Secondly, using an attenuated virus vector promotes activation of the immune system, permeabilisation of the BBB, and mounting of an adaptive immune response. While this immune response will be targeted primarily against the attenuated virus vector the infiltration of antibody-producing B cells will also enable the clearance of pathogenic RABV from the CNS.

## 5.2 RESULTS

### 5.2.1 Toxicity assessment of RABV-62scFv

Two routes of administration were used to assess RABV-62scFv *in vivo* pathogenicity: intracranial and intravenous. Intracranial administration of RABV-62scFv was chosen as it represented the fastest method of delivering RABV-62scFv behind the BBB. iv inoculation was assessed as this represented a less invasive route of infection which would be ideal for downstream applications. Here (Figure 5:1), Balb/c mice were infected either ic or iv with four different doses ( $10^1$ ,  $10^2$ ,  $10^3$ , or  $10^4$  FFU/dose) of either RABV-62scFv or a lethal positive control virus, RABV-CVS-mCH. A cell culture media control [minimum essential media (MEM), Gibco] was included to control for complications of ic/iv inoculations. Mice were observed until the development of clinical symptoms (score 1) where they were humanely terminated. Table 5:1 shows the symptom severity scoring system used in this experiment.

| Clinical Score | Clinical Symptoms                                           | Action                         |
|----------------|-------------------------------------------------------------|--------------------------------|
| 0              | No effect                                                   | Monitor as standard            |
| 1              | Ruffled fur<br>Hunched back<br>Paralysis in inoculated. leg | All mice terminated by score 1 |
| 2              | Affected gait<br>(slow or circular movements)               | All mice terminated by score 1 |
| 3              | Hind quarter Paralysis                                      | All mice terminated by score 1 |
| 4              | Progressive paralysis                                       | All mice terminated by score 1 |

|   |                                                   |                                |
|---|---------------------------------------------------|--------------------------------|
| 5 | Prostration, permanent<br>recumbency<br><br>Death | All mice terminated by score 1 |
|---|---------------------------------------------------|--------------------------------|

Table 5:1: The ethical end point document used to score severity of RABV infection symptoms. Mice would progress from score 1 to score 5. In this study, as any indication of RABV infection would mean that mice should be terminated the clinical endpoint for this study was determined as clinical score 1.

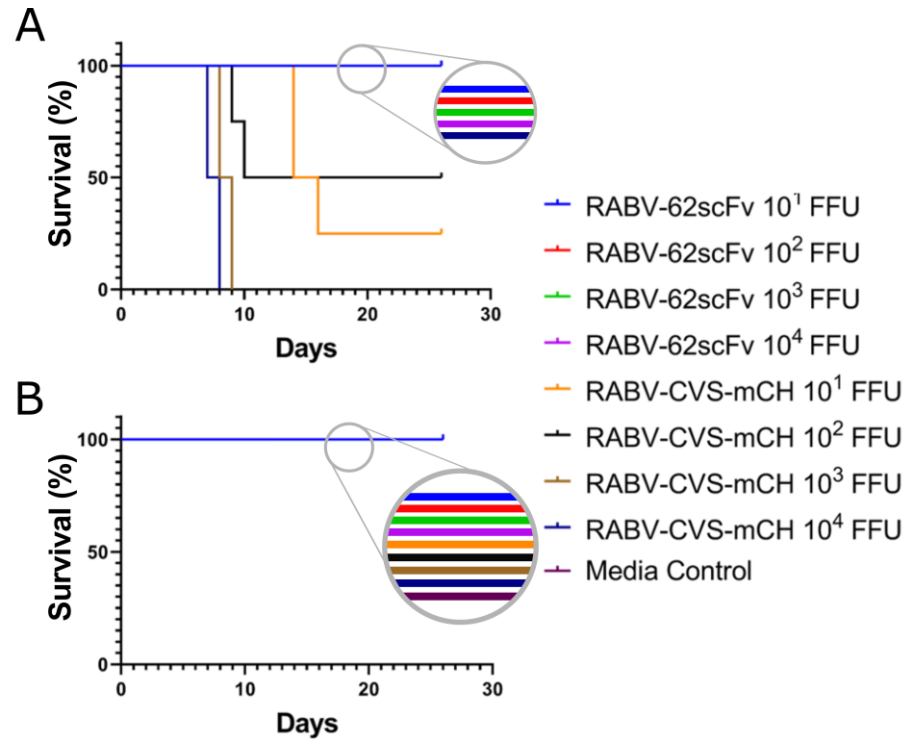


Figure 5:1: Survival curves of mice used to assess RABV-62scFv toxicity. Mice were given RABV-62scFv, RABV-CVS-mCH, or a media control either ic (A) or iv (B). Mice were then observed for 28 days for the development of clinical symptoms where they would be humanely sacrificed.  $10^1$ ,  $10^2$ ,  $10^3$ , or  $10^4$  FFU of virus (or media control) in 30  $\mu$ L was delivered by needle either into the tail vein (iv) or brain (ic) of 3-4-week-old Balb/c mice.

As shown in Figure 5:1, as expected, all mice given the media control either ic or iv survived. Furthermore, when mice were given RABV-CVS-mCH ic (A), 100% mortality was observed at the doses of  $10^3$ - $10^4$  FFU, and there were 25 and 50% survival at doses  $10^1$  and  $10^2$  FFU, respectively. Therefore, as mortality after ic inoculation is indicative of successful infection of the mouse brain, these results demonstrated that the ic route was effective, in this experiment, in delivering RABV directly to the brain. This was in contrast to mice delivered virus by the iv route. Regardless of dose, mice given RABV-CVS-mCH did not develop symptoms

and all survived until the end of the experiment. Most importantly, regardless of route of administration or dose given, all mice administered with RABV-62scFv survived until the end of the study and did not develop symptoms. In the case of iv administration, lack of mortality may be due to an inability of RABV to properly initiate infection (as inferred by 100% survival in RABV-CVS-mCH challenged mice). However, the presence of mortality in ic RABV-CVS-mCH challenged mice indicated that the ic route was effective in allowing CNS and brain infection.

#### 5.2.1.1 Serological assessment of mice infected with RABV-62scFv

After mice were humanely sacrificed, either at the end of the study or at the development of clinical symptoms, mice were cardiac punctured, and serum separated from whole blood. A modified fluorescent antibody virus neutralisation test (mFAVN, Section 2.17) was then performed to assess antibody titre. Furthermore, as mFAVN was performed using the virus each mouse was infected with, and due to the presence of the K226R mutation in the glycoprotein of RABV-62scFv, the antibodies measured by the mFAVN are of host-origin, rather than 62-71-3 scFv produced by RABV-62scFv infection. The results of the mFAVN are shown in Figure 5:2.

As shown in Figure 5:2A, anti-RABV antibodies were present in high levels in all mice infected ic with RABV-62scFv regardless of dose. Furthermore, only in mice given the highest titre of RABV-CVS-mCH were anti-RABV antibodies detected. Figure 5:2B shows the results on mFAVN performed on sera from mice infected iv with either RABV-62scFv or RABV-CVS-mCH or given a media control. As shown, presence of anti-RABV antibodies correlated with dose of RABV-62scFv given, with antibody levels below the cut-off for detection in mice given  $10^1$  FFU RABV-62scFv. Antibodies were also present in mice given  $10^{2/3/4}$  FFU of RABV-CVS-mCH, however, no correlation between dose given and anti-RABV antibody titre was observed.

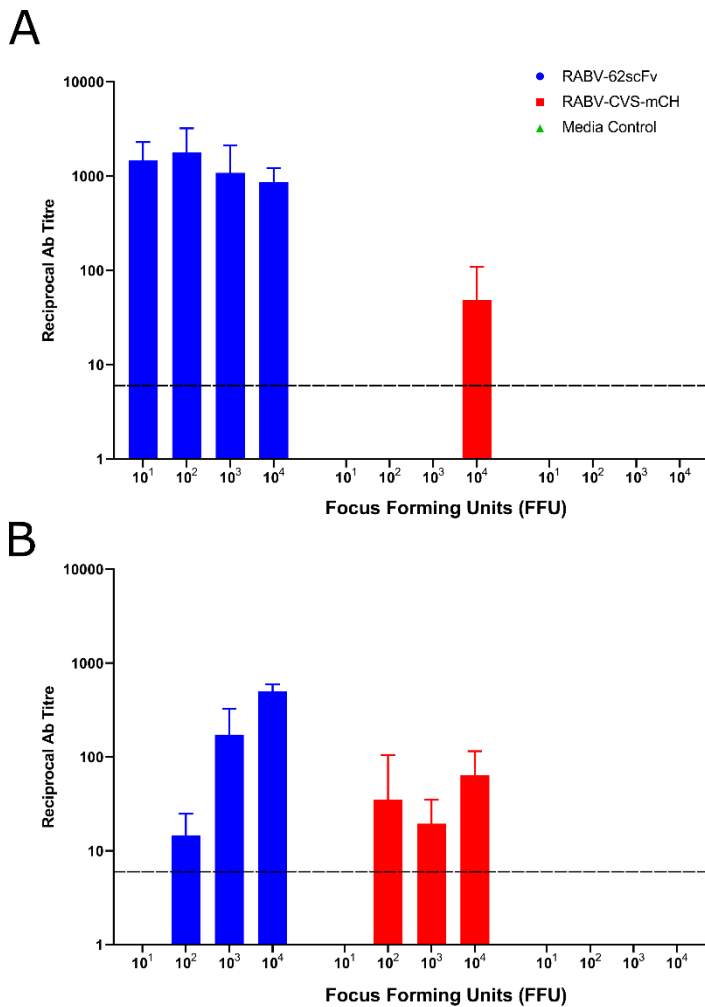


Figure 5:2: Serological assessment of mouse sera after challenge (ic or iv) with either RABV-62scFv or RABV-CVS-mCH, or media control. A, shows results from mice challenged ic while B, shows results from mice challenged iv. A modified fluorescent antibody virus neutralisation (FAVN) test was performed to assess levels of neutralising anti-RABV antibody in mouse blood. In these tests, RABV-62scFv or RABV-CVS-mCH was used to assess neutralising antibody level in the sera of mice challenged with those viruses, respectively. This meant that, in the case of RABV-62scFv challenged mice, only antibodies of host origin were measured and not scFv produced by RABV-62scFv cellular infection. Sera from mice given a media control was tested for neutralisation against RABV-CVS-mCH. Dotted line represents the cutoff for detection at a reciprocal antibody titre of 7.49.

#### 5.2.1.2 Investigating the presence of RABV N protein and live virus in mouse brain

To assess the ability of the host immune response to clear RABV virus from the brain of infected mice, after mice were humanely terminated (either at the development of symptoms or at the end of the study) a fluorescent antibody test (FAT, Section 2.18) and rabies tissue culture infection test (RTCIT, Section 2.19) was performed on brain smears and brain

homogenate to visualise RABV N protein and isolate live virus, respectively. Table 5:2 shows the results of these tests.

| Virus challenge group | Dose (FFU)                       | Challenge route | Survival to the end of the study | Presence of RABV N protein in brain smear (FAT) | Presence of live virus in brain homogenate (RTCIT) |
|-----------------------|----------------------------------|-----------------|----------------------------------|-------------------------------------------------|----------------------------------------------------|
| RABV-62scFv           | 10 <sup>1</sup> -10 <sup>4</sup> | IC              | Y                                | -                                               | -                                                  |
| RABV-CVS-mCH          | 10 <sup>1</sup>                  |                 | N                                | +                                               | +                                                  |
|                       |                                  |                 | N                                | +                                               | +                                                  |
|                       |                                  |                 | N                                | +                                               | +                                                  |
|                       |                                  |                 | Y                                | -                                               | -                                                  |
| RABV-CVS-mCH          | 10 <sup>2</sup>                  |                 | Y                                | -                                               | -                                                  |
|                       |                                  |                 | Y                                | -                                               | -                                                  |
|                       |                                  |                 | N                                | +                                               | +                                                  |
|                       |                                  |                 | N                                | +                                               | +                                                  |
|                       | 10 <sup>3</sup> -10 <sup>4</sup> |                 | N                                | +                                               | +                                                  |
| RABV-62scFv           | 10 <sup>1</sup> -10 <sup>4</sup> | IV              | Y                                | -                                               | -                                                  |
| RABV-CVS-mCH          | 10 <sup>1</sup> -10 <sup>4</sup> |                 | Y                                | -                                               | -                                                  |
| Media control         | N/A                              | IC / IV         | Y                                | -                                               | -                                                  |

Table 5:2: Description of the dose, challenge route, survival, presence of N protein and live virus in mice used to assess RABV-62scFv toxicity. To assess the presence of either RABV nucleoprotein (N) or live RABV in the brains of challenged mice, either a fluorescent antibody test (FAT) or rabies tissue culture infection test (RTCIT) was performed, respectively. This table details the dose and virus each experimental group was given and the route of administration. As some mice survived till the end of the study and some developed symptoms of RABV infection, this is also illustrated. As results from FAT or RTCIT indicate either the presence of absence of RABV N protein or live RABV, results have been denoted with either a + (positive) or – (negative).

As shown in Table 5:2, regardless of group, dose, or challenge route, in all mice which developed symptoms of RABV, FAT and RTCIT revealed presence of RABV N protein and live virus in brain smears and homogenate, respectively. Similarly, brains of mice which survived the duration of the experiment did not contain either RABV N protein or live virus. This indicates that RABV-CVS-mCH was successful in both infecting and replicating within the brain after ic infection, eventually resulting in symptom onset. In contrast, when RABV-62scFv was delivered ic to mice, the host immune system was able to clear virus and prevent the onset of symptoms. These data also support the hypothesis that iv administered RABV is unable to infect the CNS of inoculated mice, as all iv inoculated mice both survived infection and were both FAT and RTCIT negative.

### 5.2.1.3 Histopathological assessment of CNS immune cell infiltration

Observations from Sections 5.2.1.1 and 5.2.1.2 suggest that upon ic inoculation, RABV-62scFv was able to permeabilise the host BBB and activate the host immune system to allow clearance of RABV-62scFv from the brain and CNS. To investigate this, mouse brains were formalin fixed and paraffin embedded for histopathological investigation. Histopathological brain sections were taken from mice inoculated with RABV-CVS-mCH by the ic route. This was because mice inoculated with RABV-62scFv survived until the end of the experiment (Day 28) with no RABV N protein present (Table 5:2) in brain smears and were, therefore, deemed unlikely to show the presence of infiltrating immune cells. Sections of RABV-CVS-mCH-infected mouse brain were stained for RABV N protein and T cells (CD3) (See Section 2.26.2). Images from these experiments are shown in Figure 5:3.

As shown in Figure 5:3A and B, within the hippocampus there was Infrequent but focal areas of RABV N protein labelling. Furthermore, neighbouring areas were associated with rare CD3+ cells presence within the hippocampal laminar (1) and neuropil (2 and 3). In midbrain sections (C and D), scattered neuronal RABV N protein labelling was present with rare and dispersed CD3 presence. Lastly, while presence of CD3 was absent (image not shown), E shows a focal area within the thalamus with neuronal cells and neuropil labelled for RABV N protein.

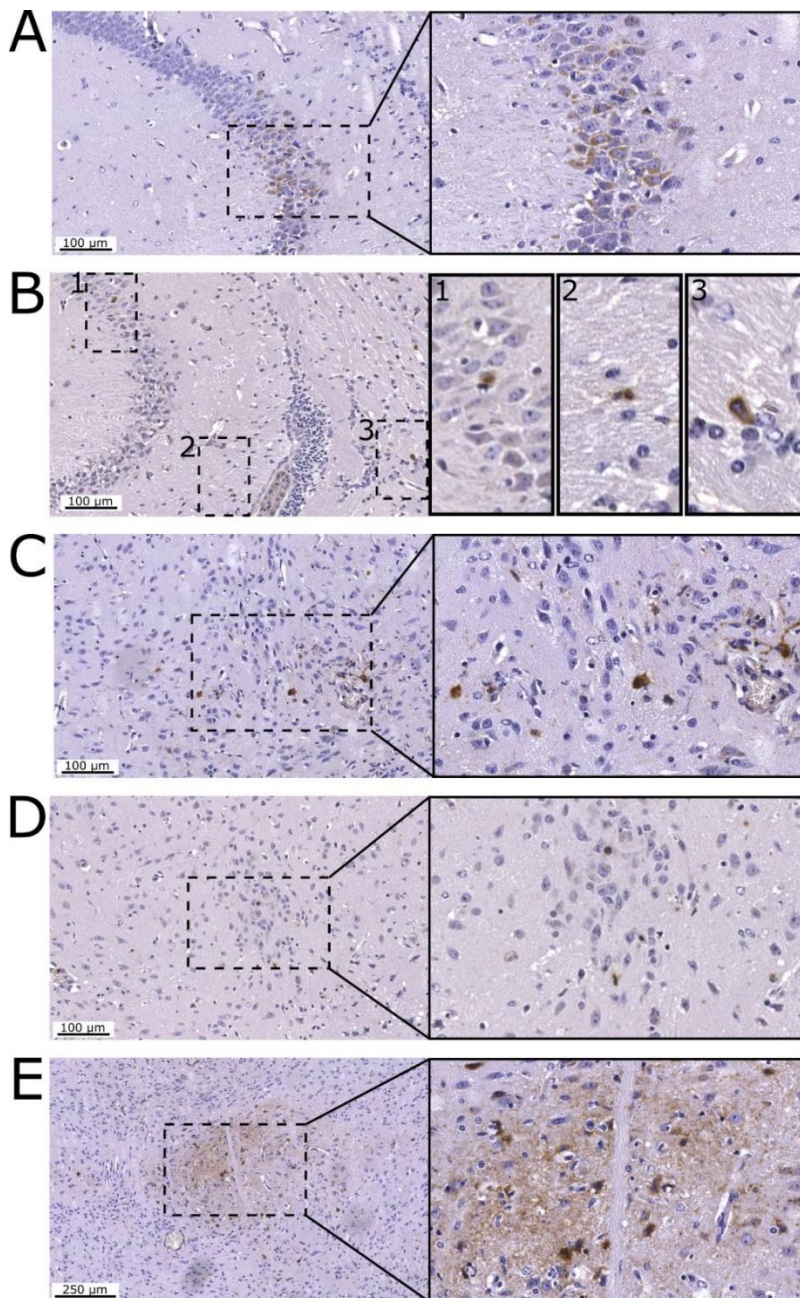


Figure 5:3: Histopathological assessment of mouse brains after ic challenge with RABV-CVS-mCH. Images A to E show representative images of sections from a mouse brain. Left hand side images have been taken at 200x magnification (A-D) or 100x magnification (E), with the right-hand side images showing magnification of the areas bordered by the dotted boxes in the left-hand side images at 400x resolution. A and B show the hippocampus region, where brown staining for the RABV N protein is shown in A and staining for CD3 is shown in B. Images C and D show midbrain sections where brown staining indicates presence of RABV N protein and CD3 respectively. Lastly, E shows a section of the thalamus where brown staining indicates RABV N staining.

### 5.2.2 Assessment of RABV-62scFv as a post-exposure treatment

Section 5.2.1 revealed that RABV-62scFv is severely attenuated and is, therefore, non-lethal upon ic inoculation of mice. Furthermore, RABV-62scFv is effective in permeabilising the BBB and promoting the infiltration of B cells, T cells, and macrophages into the brain/CNS which remain up to 28 days after challenge. To assess the ability of RABV-62scFv to act as a post-exposure treatment and prevent the onset of clinical disease after lethal challenge, a second *in vivo* study was performed. Here, mice were first challenged by two im doses of lethal rabies virus RV437 ( $10^3$  FFU per dose) into both hind legs. At either day 0, three, or five, post-challenge, mice were then treated with  $10^4$  FFU of RABV-62scFv ic. Mice were observed for 28 days after challenge and humanely sacrificed when symptom severity reached previously defined ethical cut-off points (score 3). The scoring system used in this experiment is shown in Table 5:3

| Clinical Score | Clinical Symptoms                                                                   | Action                                 |
|----------------|-------------------------------------------------------------------------------------|----------------------------------------|
| 0              | No effect                                                                           | Monitor as standard                    |
| 1              | Ruffled fur<br>Hunched back<br>Limping in inoculated leg(s)                         | Monitor                                |
| 2              | Severe limping in inoculated legs but still able to reach food/water<br>Weight loss | Monitor                                |
| 3              | Hind quarter Paralysis<br>Inability to reach food/water                             | Terminate                              |
| 4              | Progressive paralysis                                                               | All mice will be terminated by score 3 |
| 5              | Prostration, permanent recumbency<br>Death                                          | All mice will be terminated by score 3 |

Table 5:3: Description of ethical endpoints used to assess use of RABV-62scFv as a post-exposure treatment. To assess whether mice had reached ethical end points, symptoms of RABV infection were mapped to a clinical score sheet. As this experiment was aimed at assessing the use of RABV-62scFv as a post-exposure prophylaxis treatment, the ethical endpoint for this study was set at clinical score 3. Furthermore, as mice were lethally

challenged by dual im injection, and not ic as before, the definitions of each clinical score were altered to reflect this.

Shown below, Figure 5:4 illustrates the survival rates of mice used to assess the effectiveness of RABV-62scFv as a post-exposure prophylaxis. As expected, all mice in the treatment-only group (single ic dose of RABV-62scFv) survived. All mice in the RV437 challenge-only group developed symptoms by day eight, and all were terminated by day 15. Of the mice which were treated with RABV-62scFv alongside RV437 lethal challenge, mice began to show symptoms of RABV infection by day 10 and mice which progressed to ethical end points were terminated by day 17. Interestingly, in this group, not all mice which developed symptoms progressed to ethical end points. Of the five mice which showed signs of RABV infection, one mouse recovered after two days, and another mouse began showing symptoms on day 18 but symptoms did not progress over the course of the experiment. In mice which were treated on day three after dual im RV437 challenge, similar results were seen. Firstly, only 33% of this group did not show symptoms and progressed to symptomatic end points. Interestingly, one of these mice, similar to observations of mice treated on day 0, began displaying symptoms of RABV infection on day 16, however, did not progress and survived until the end of the study. Of the mice which were terminated, symptom onset was seen earliest at day 11 and all mice were terminated by day 16. Lastly, in mice which were treated on day five after RV437 challenge, a 0% survival rate was observed. Here, symptom onset began from day nine, and all mice were terminated by day 22.

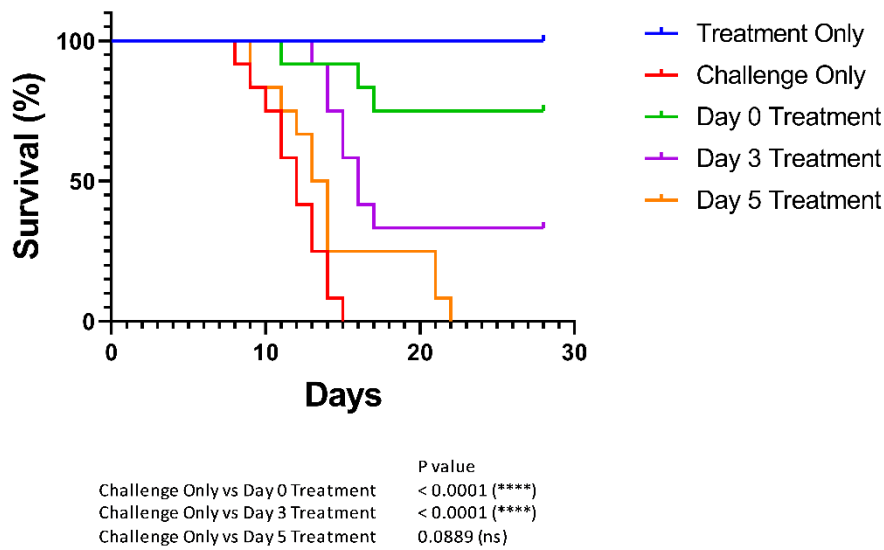
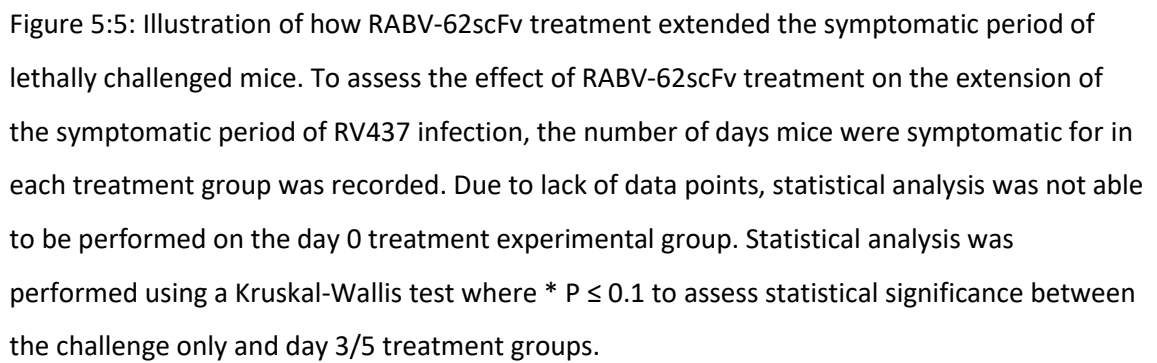


Figure 5:4: A survival curve of mice used to investigate the applicability of RABV-62scFv as a post-exposure prophylaxis treatment. Five experimental groups existed within this study, mice were either administered with treatment (RABV-62scFv) or challenge (RV437) virus only, or administered with challenge virus on day 0 and treated on day 0, 3, or 5. Mice were then observed for the development of clinical symptoms and terminated upon reaching ethical endpoints. A Log-rank (Mantel-Cox) test was used to determine significant differences between the challenge only group and the experimental treatment groups where \*\*\*\*  $P \leq 0.0001$ .

Throughout this study, it was observed that mice allocated to treatment groups were symptomatic for longer than mice in the challenge-only groups. To assess this, the number of days between symptom onset and termination was recorded and the results shown in Figure 5:5. Unfortunately, as data points are only available from mice which were terminated, statistical analysis was not able to be performed between the challenge-only group and the day 0 treatment group. However, as shown in Figure 5:5, a statistically significant (\*) increase in the number of days mice remained symptomatic in the day three treatment group compared to the challenge-only group was observed. This significant difference was not present when comparing the day five treatment group to the challenge only group.



Serological response to RABV infection (either RABV-62scFv or RV437) in mice was assessed by FAVN. Mice that were given only RV437 or RABV-62scFv (challenge-only and treatment-only groups) were assessed only for response against RV437 or RABV-62scFv, respectively. Mice that were given both RV437 and RABV-62scFv were assessed for neutralising antibody against both viruses. Table 5:4 comprehensively illustrates serological titres of mice against RABV-62scFv and RV437 within the context of the progression of clinical scores after lethal challenge and treatment.

| Challenge Only | Mouse # | Days post-lethal RV437 challenge |   |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    | Reciprocal<br>titre against |              |
|----------------|---------|----------------------------------|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|-----------------------------|--------------|
|                |         | 0                                | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28                          | <u>RV437</u> |
|                | 1       | 0                                | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1  | 1  | 2  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |                             | 140.3        |
|                | 2       | 0                                | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  |    |                             | 81.0         |
|                | 3       | 0                                | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0  | 1  | 2  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |                             | 46.8         |
|                | 4       | 0                                | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0  | 1  | 1  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |                             | 46.8         |
|                | 5       | 0                                | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0  | 0  | 1  | 2  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |                             | 140.3        |
|                | 6       | 0                                | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0  | 0  | 0  | 1  | 1  | 1  |    |    |    |    |    |    |    |    |    |    |    |    |                             | 46.8         |
|                | 7       | 0                                | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0  | 0  | 0  | 0  | 1  | 2  |    |    |    |    |    |    |    |    |    |    |    |    |                             | 140.3        |
|                | 8       | 0                                | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |                             | 0.0          |
| 9              | 0       | 0                                | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0  | 1  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    | 15.6                        |              |
| 10             | 0       | 0                                | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 2  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    | 32.4                        |              |
| 11             | 0       | 0                                | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    | 0.0                         |              |
| 12             | 0       | 0                                | 0 | 0 | 0 | 0 | 0 | 0 | 1 |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    | N/A                         |              |

| Treatment Only | Days post-lethal RV437 challenge |   |   |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    | Reciprocal<br>titre against<br><b>RABV-62scFv</b> |    |       |
|----------------|----------------------------------|---|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|---------------------------------------------------|----|-------|
|                | Mouse #                          | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27                                                | 28 |       |
|                |                                  |   |   |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |                                                   |    |       |
|                | 1                                | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0                                                 | 0  | 420.9 |
|                | 2                                | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0                                                 | 0  | 420.9 |
|                | 3                                | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0                                                 | 0  | 46.8  |
|                | 4                                | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0                                                 | 0  | 729.0 |
|                | 5                                | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0                                                 | 0  | 576.0 |
|                | 6                                | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0                                                 | 0  | 203.6 |
|                | 7                                | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0                                                 | 0  | 106.1 |
|                | 8                                | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0                                                 | 0  | 101.8 |
|                | 9                                | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0                                                 | 0  | 420.9 |
|                | 10                               | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0                                                 | 0  | 140.3 |
|                | 11                               | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0                                                 | 0  | 729.0 |
|                | 12                               | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0                                                 | 0  | 140.3 |

| Day 0 Treatment | Days post-lethal RV437 challenge |   |   |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    | Reciprocal titre against |    |             |        |        |
|-----------------|----------------------------------|---|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|--------------------------|----|-------------|--------|--------|
|                 | Mouse #                          | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27                       | 28 | RABV-62scFv | RV437  |        |
|                 | 1                                | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0                        | 0  | 288.0       | 243.0  |        |
|                 | 2                                | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0                        | 0  | 407.3       | 140.3  |        |
|                 | 3                                | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0                        | 0  | 50.9        | 46.8   |        |
|                 | 4                                | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0                        | 0  | 203.6       | 81.0   |        |
|                 | 5                                | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1                        | 1  | 1           | 4608.0 | 2187.0 |
|                 | 6                                | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0  | 1  | 1  | 1  | 1  | 1  | 2  |    |    |    |    |    |    |    |    |    |    |                          |    |             | 101.8  | 140.3  |
|                 | 7                                | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0                        | 0  | 0           | 101.8  | 140.3  |
|                 | 8                                | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0                        | 0  | 0           | 407.3  | 67.4   |
| 9               | 0                                | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0                        | 0  | 407.3       | 140.3  |        |
| 10              | 0                                | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1  | 2  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |                          |    | 576.0       | 81.0   |        |
| 11              | 0                                | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0  | 0  | 0  | 0  | 0  | 0  | 1  | 1  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0                        | 0  | 814.6       | 420.9  |        |
| 12              | 0                                | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0  | 0  | 0  | 0  | 0  | 1  | 1  | 1  |    |    |    |    |    |    |    |    |    |                          |    | 407.3       | 420.9  |        |

|                 | Days post-lethal RV437 challenge |   |   |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    | Reciprocal titre against |             |        |
|-----------------|----------------------------------|---|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|--------------------------|-------------|--------|
|                 | Mouse #                          |   |   |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |                          | RABV-62scFv | RV437  |
|                 |                                  | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27                       |             |        |
| Day 3 Treatment | 1                                | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0  | 1  | 1  | 2  |    |    |    |    |    |    |    |    |    |    |    |    |    |                          | 407.3       | 729.0  |
|                 | 2                                | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0  | 0  | 1  | 1  | 1  | 2  |    |    |    |    |    |    |    |    |    |    |    |                          | 424.3       | 785.8  |
|                 | 3                                | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0                        | 407.3       | 140.3  |
|                 | 4                                | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0  | 0  | 0  | 0  | 0  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1                        | 1629.2      | 2187.0 |
|                 | 5                                | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0                        | 1629.2      | 3788.0 |
|                 | 6                                | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0                        | 3258.3      | 3788.0 |
|                 | 7                                | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0  | 1  | 1  | 1  | 2  |    |    |    |    |    |    |    |    |    |    |    |    |                          | 203.6       | 420.9  |
|                 | 8                                | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0  | 0  | 0  | 0  | 0  | 0  | 1  |    |    |    |    |    |    |    |    |    |    |                          | 424.3       | 1262.7 |
|                 | 9                                | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0  | 1  | 1  | 1  | 3  |    |    |    |    |    |    |    |    |    |    |    |    |                          | N/A         | N/A    |
|                 | 10                               | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0  | 1  | 1  | 1  | 1  | 1  | 1  |    |    |    |    |    |    |    |    |    |    |                          | 407.3       | 420.9  |
|                 | 11                               | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0  | 1  | 1  | 1  | 1  | 1  | 3  |    |    |    |    |    |    |    |    |    |    |                          | 203.6       | 140.3  |
|                 | 12                               | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0  | 1  | 1  | 1  | 1  | 1  |    |    |    |    |    |    |    |    |    |    |    |                          | 848.5       | 420.9  |
| Day 5 Treatment | 1                                | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0  | 0  | 0  | 1  | 2  |    |    |    |    |    |    |    |    |    |    |    |    |                          | 203.6       | 140.3  |
|                 | 2                                | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0  | 0  | 1  | 1  | 2  |    |    |    |    |    |    |    |    |    |    |    |    |                          | 848.5       | 1262.7 |
|                 | 3                                | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0  | 1  | 1  | 2  |    |    |    |    |    |    |    |    |    |    |    |    |    |                          | 25.5        | 15.6   |
|                 | 4                                | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0  | 0  | 1  | 0  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  |    |    |    |    |    |                          | N/A         | N/A    |
|                 | 5                                | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |                          | 6.4         | 0.0    |
|                 | 6                                | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0  | 0  | 0  | 0  | 1  | 1  | 1  | 1  | 1  | 1  | 2  |    |    |    |    |    |    |                          | 814.6       | 1262.7 |
|                 | 7                                | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 2  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |                          | 407.3       | 140.3  |
|                 | 8                                | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |                          | 26.5        | 15.6   |
|                 | 9                                | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0  | 0  | 1  | 2  |    |    |    |    |    |    |    |    |    |    |    |    |    |                          | 26.5        | 15.6   |
|                 | 10                               | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0  | 0  | 0  | 0  | 1  | 1  | 1  | 2  | 1  | 1  | 1  | 2  |    |    |    |    |    |                          | 212.1       | 420.9  |
|                 | 11                               | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1  | 1  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |                          | 106.1       | 140.3  |
|                 | 12                               | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1  | 1  | 1  | 2  |    |    |    |    |    |    |    |    |    |    |    |    |    |                          | 848.5       | 1262.7 |

Table 5:4: Reciprocal serological titres and clinical scores of mice in each experimental group in the in vivo study assessing RABV-62scFv as a post-exposure treatment. Mice were observed twice daily and assigned a clinical score according to Table 5:3 (clinical score 0=green, 1 and above=yellow). Upon reaching clinical score 2 or above, most mice were terminated and cardiac punctured. Sera was assessed for neutralising antibody against RABV-62scFv and/or RV437 where appropriate and the reciprocal titres shown. Reciprocal titre values against RABV-62scFv and RV437 have been colour graded according to magnitude.

#### 5.2.2.2 Histopathological assessment of N protein presence in mice

Either at the end of the study described in Section 5.2.2, or when mice reached clinical endpoints as described in Table 5:3, a selection of mice were fixed in formalin for

histopathological assessment (See Section 2.16). Specifically, a single representative mouse was chosen from the treatment only group and three mice from the challenge only group. Two mice were chosen from the day 0 treatment group, and four from the day three and five treatment groups. These mice were assessed for both RABV N protein and pathological lesion presence in their cerebrum, hippocampus, thalamus, midbrain, cerebellum, brain stem, and spinal cord. The results from histopathological analysis are shown in Table 5:5 and images shown in Figure 5:6 and Figure 5:7.

As shown, RABV N protein was present throughout the cerebrum, hippocampus, thalamus, midbrain, cerebellum, brain stem, and spinal cord in mice which succumbed to RABV infection in the Challenge only group with lesions detected in the midbrain, cerebellum, and brain stem of mouse five. Two mice were selected from the day 0 treatment group, one which was culled (mouse six) and one which survived until the end of the experiment (mouse nine). As expected, mouse nine did not reveal presence of either lesions or RABV N protein. However, mouse 6, which was culled, did not show presence of RABV N protein or lesions. This will be discussed later (Section 5.3). Mice which succumbed to RABV infection within the day three treatment group displayed presence of N protein throughout various sections in various proportions with lesions present in the brain stem of mice one and 11, while mouse six (which survived until the end of the experiment) was negative for RABV N protein in all sections. Mice of the last treatment group (day five treatment) all succumbed to infection, with histopathology revealing presence of RABV N protein and lesions in multiple sections.

| Location                     | Cerebrum |    | Hippocampus |    | Thalamus |    | Midbrain |    | Cerebellum |    | Brain Stem |    | Spinal Cord |    | Clinical Score                                                        |
|------------------------------|----------|----|-------------|----|----------|----|----------|----|------------|----|------------|----|-------------|----|-----------------------------------------------------------------------|
| Presence of N or Lesion (Ls) | N        | Ls | N           | Ls | N        | Ls | N        | Ls | N          | Ls | N          | Ls | N           | Ls |                                                                       |
| Treatment only Mouse 8       | 0        | 0  | 0           | 0  | 0        | 0  | 0        | 0  | 0          | 0  | 0          | 0  | 0           | 0  | Survived, no symptoms                                                 |
| Challenge only - Mouse 4     | 3        | 0  | 1           | 0  | 3        | 0  | 2        | 0  | 4          | 0  | 4          | 0  | 4           | 0  | Culled on day 14, displayed bilateral hind leg paralysis for 3.5 days |
| Challenge Only - Mouse 5     | 2        | 0  | 2           | 0  | 4        | 0  | 3        | 1  | 3          | 2  | 4          | 2  | 4           | 0  | Culled on day 13, displayed bilateral hind leg paralysis for 1.5 days |
| Challenge only - Mouse 10    | 2        | 0  | 0           | 0  | 1        | 0  | 1        | 0  | 2          | 0  | 2          | 0  | 4           | 0  | Culled on day 11, bilateral hind leg paralysis for 1.5 days           |
| Treatment day 0 - Mouse 6    | 0        | 0  | 0           | 0  | 0        | 0  | 0        | 0  | 0          | 0  | 0          | 0  | n.s.        | 0  | Culled on day 16, bilateral hind leg paralysis for 5.5 days           |
| Treatment day 0 - Mouse 9    | 0        | 0  | 0           | 0  | 0        | 0  | 0        | 0  | 0          | 0  | 0          | 0  | n.s.        | 0  | Survived, no symptoms                                                 |
| Treatment day 3 - Mouse 1    | 0        | 0  | 0           | 0  | 2        | 1  | 2        | 0  | 3          | 0  | 4          | 1  | 2           | 0  | Culled on day 13, bilateral hind leg paralysis for 2.5 days           |
| Treatment day 3 - Mouse 6    | 0        | 0  | 0           | 0  | 0        | 0  | n.s.     | 0  | 0          | 0  | 0          | 0  | 0           | 0  | Survived, no symptoms                                                 |
| Treatment day 3 - Mouse 7    | 2        | 0  | 1           | 0  | 1        | 0  | 1        | 0  | 2          | 0  | 4          | 0  | n/a         | 0  | Culled on day 13, bilateral hind leg paralysis for 3.5 days           |
| Treatment day 3 - Mouse 11   | 0        | 0  | 0           | 0  | 0        | 0  | 0        | 0  | 1          | 0  | 2          | 1  | 2           | 0  | Culled on day 16, bilateral hind leg paralysis for 3.5 days           |
| Treatment day 5 - Mouse 1    | 2        | 0  | 1           | 0  | 2        | 0  | 2        | 2  | 2          | 0  | 3          | 1  | 3           | 0  | Culled on day 13, bilateral hind leg paralysis for 1.5 days           |
| Treatment day 5 - Mouse 6    | 0        | 0  | 1           | 0  | 2        | 0  | 2        | 0  | 1          | 0  | 3          | 1  | 3           | 0  | Culled on day 21, bilateral hind leg paralysis for 7 days             |
| Treatment day 5 - Mouse 8    | 2        | 0  | 0           | 0  | 0        | 0  | 2        | 0  | 2          | 0  | 3          | 1  | 4           | 0  | Culled on day 9, bilateral hind leg paralysis for 1 days              |
| Treatment day 5 - Mouse 9    | 0        | 0  | 1           | 0  | 3        | 0  | 3        | 1  | 3          | 0  | 4          | 2  | 3           | 0  | Culled on day 14, bilateral hind leg paralysis for 1.5 days           |

Table 5:5: Results of histopathological analysis of mouse brains and spinal cord for the presence of RABV N protein or pathological lesions (Ls). Here, mice were selected from the Treatment only, Challenge only, or day 0/3/5 treatment groups where the mouse number reflects the mouse numbers given in Table 5:4. After staining for RABV N protein or assessing for presence of lesions, mouse sections were designated a number from 0 to five to indicate how widespread N protein staining or lesions were, where 1= absent, 2= scattered, 3= mild, 4=moderate, n.s.= non-specific, n/a= not available. The last column indicates when each mouse was culled (or whether it survived till the end of the experiment), and the major symptoms displayed prior to culling.

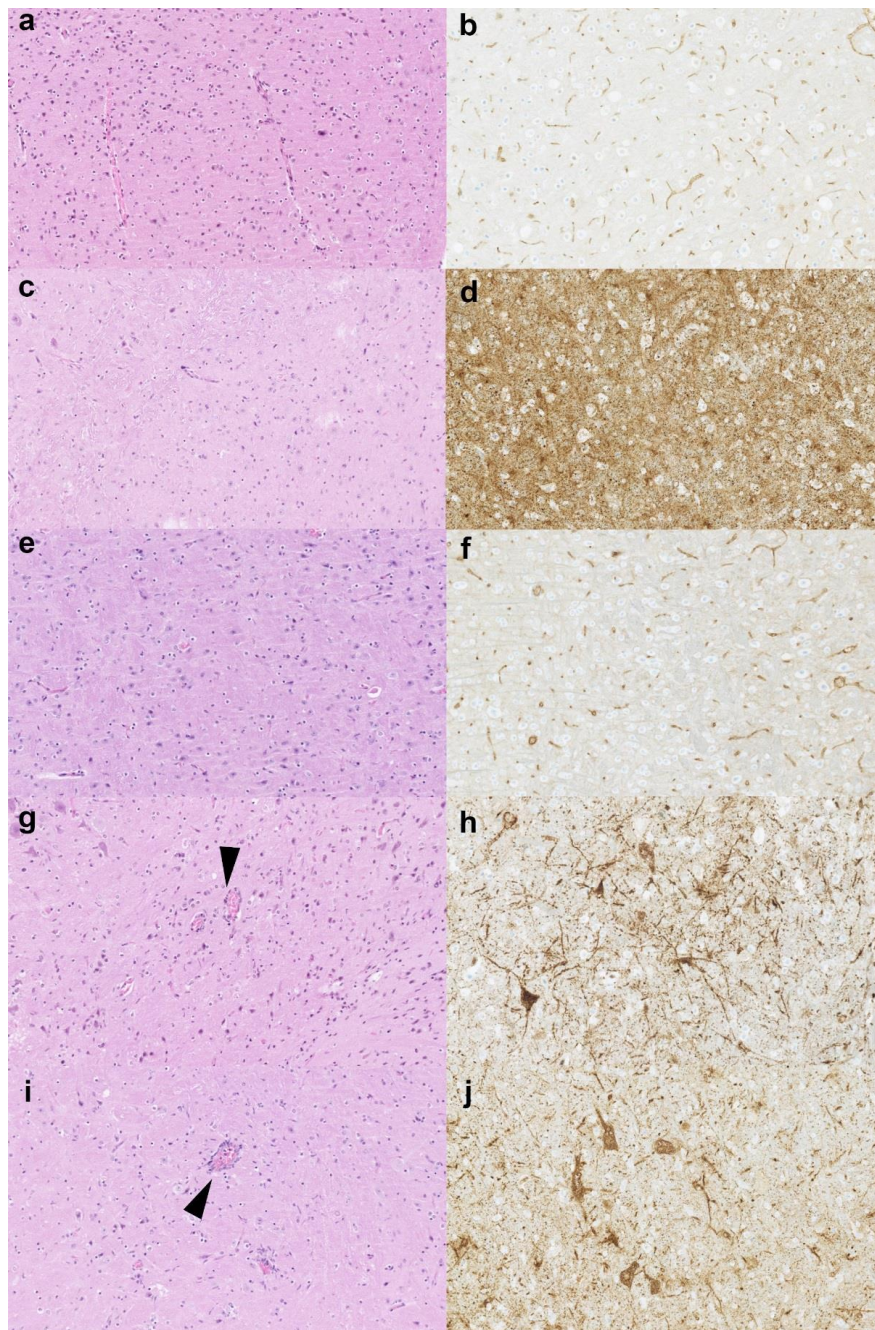


Figure 5:6: Representative images of histopathology and immunohistochemistry on brainstem of mice used to assess RABV-62scFv as a post-exposure prophylaxis treatment. Images taken at 100x and are of serial sections of H&E stain (first column) and anti-RABV N protein IHC (second column). a/b show images from the treatment only group (mouse 8). c/d show images from the challenge-only group (mouse 4), e/f show images from the day 0 treatment group (mouse 6), g/h show images taken from the day 3 treatment group (mouse 1), and i/j show images taken from the day 5 treatment group (mouse 1). Rare vasculitis was detected in mice treated on day 0 and 3, (arrowheads, images g and i) and was associated with virus infected areas consisting of moderate abundance of virus antigens.

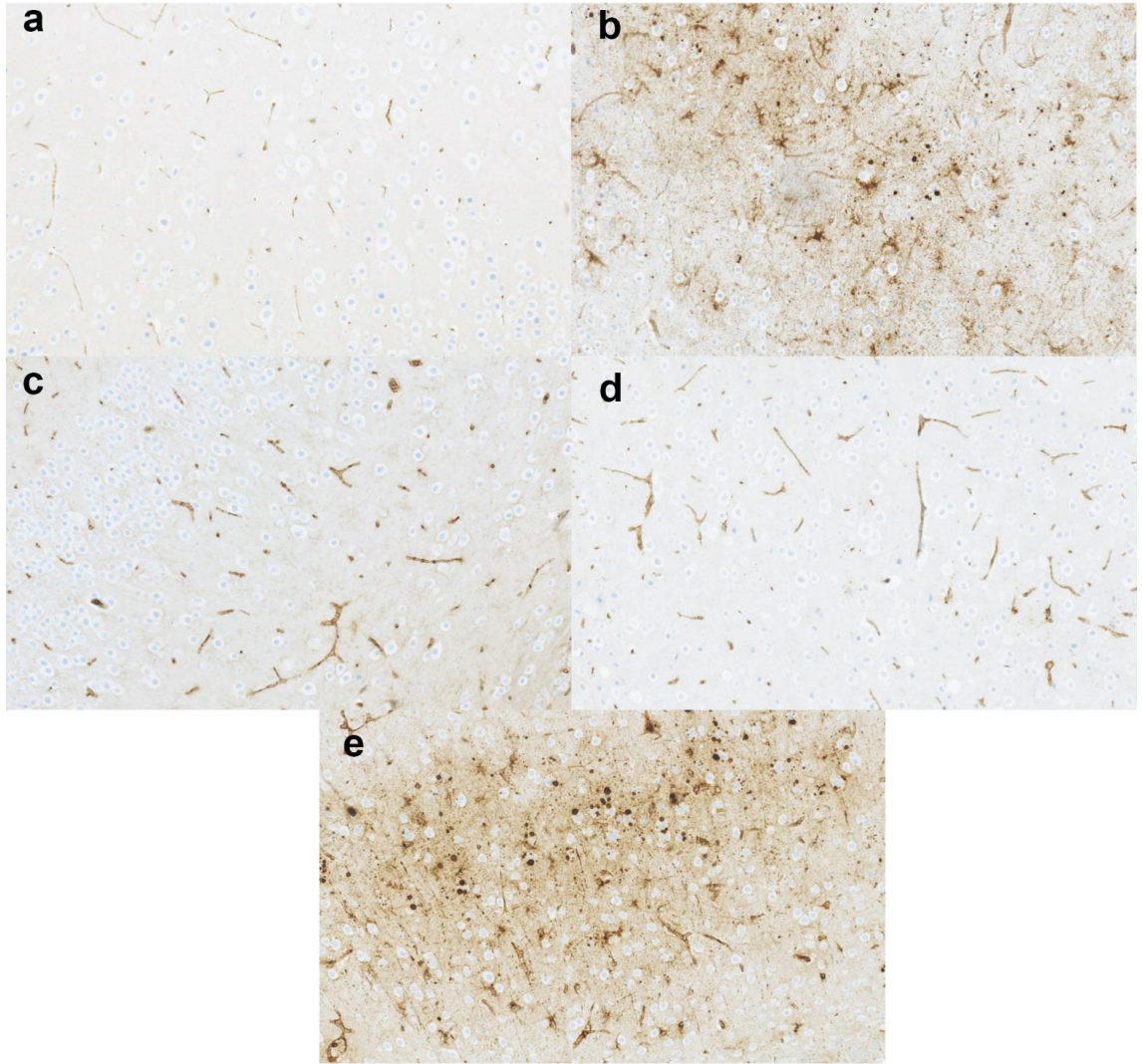


Figure 5:7: Representative images of Immunohistochemistry on the cerebrum of mice for RABV N protein. Image A shows immunohistochemistry of a mouse from the treatment only group (mouse 8), while b shows mouse 4 from the challenge only group, c shows mouse 6 from the day 0 treatment group, d shows mouse 1 from the day 3 treatment group, and e shows mouse 1 from the day 5 treatment group. As shown, virus infection was detected only in b and e. Images taken at 100x.

### 5.3 DISCUSSION

This chapter describes and explores two *in vivo* studies aimed at assessing the applicability of RABV-62scFv as a potential treatment for RABV infection. First, as RABV-62scFv is a novel recombinant virus its pathogenicity was assessed upon inoculation of an *in vivo* mouse model. As discussed in Chapter 4, experiments investigating the growth kinetics and cellular dissemination of RABV-62scFv had suggested that RABV-62scFv would be apathogenic upon *in vivo* infection. Therefore, two routes of administration were chosen to assess pathogenicity: ic and iv. ic administration is a common route of infection for RABV and remains the best route to assess pathogenicity<sup>180</sup>. For example, while some viruses may not be pathogenic when administered peripherally (for example im or by the intraperitoneal route), ic administration may result in mortality regardless<sup>240</sup>. This is because when delivered ic the virus is able to immediately replicate within the brain, circumventing any host defence mechanisms which may result in reduced pathogenicity when delivered peripherally. The other route of administration chosen, iv, was selected due to its applicability in downstream treatment studies (iv is non-invasive and a common procedure performed in humans). If iv infection was able to productively infect the CNS, by being able to cause permeabilisation of the BBB and an influx of immune effector cells, this would represent an ideal non-invasive treatment delivery route. As demonstrated in Section 5.2.1, iv RABV infection of mice was shown to be apathogenic with a positive correlation between RABV-62scFv dose and neutralising serum antibody. However, mice infected with RABV-CVS-mCH by the iv route did not show any signs of RABV infection. This suggested that RABV-CVS-mCH was not able to cross the BBB directly and infect the host CNS. Therefore, RABV-62scFv would also be unlikely to cross the BBB and cross into the CNS when administered by the iv route. This indicated that iv administration was an unsuitable route for RABV-62scFv delivery as a treatment of post-symptom onset rabies.

In contrast to iv infection, ic administration allowed effective infection of the brain and CNS. Mice infected with the positive control virus, RABV-CVS-mCH, developed clinical signs of RABV infection and were terminated appropriately. FAT and RTCIT analysis of RABV-CVS-mCH challenged mice brain also revealed the presence of RABV N protein and live virus. Serological analysis of blood taken from mice upon termination showed titres of RABV-CVS-mCH neutralising antibody similar to that found in mice challenged iv by the same virus. Similar to iv infection, RABV-62scFv delivered ic was apathogenic at all doses tested. In mice challenged ic with RABV-62scFv a strong anti-RABV-62scFv serological response was seen. This, in addition to mice brains being absent of RABV N protein or live virus at the end of the study, suggested that mice were able to effectively clear RABV-62scFv from their CNS after ic inoculation. While

a direct measurement of BBB permeability (such as injection of sodium fluorescein) was not available, histopathological examination of mouse brains was performed for detection of MAC387, CD3, CD79a, and RABV N protein. As described in Section 2.26, histopathology was unable to detect these proteins in mice administered with RABV-62scFv, while mice which displayed symptoms of RABV infection after RABV-CVS-mCH infection were positive for both RABV N protein and CD3 (often in the same area). The presence of CD3<sup>+</sup> cells within the CNS of RABV-CVS-mCH challenged mice was unsurprising. While most research has focused upon the recruitment of B cells after attenuated RABV infection, studies have shown that after lethal RABV challenge the BBB demonstrates low levels of permeabilisation as early as day six in concert with the recruitment of T cells<sup>94,367-370</sup>. While it was hypothesised that MAC387<sup>+</sup> (macrophage), CD3<sup>+</sup> (T cells), and CD79a<sup>+</sup> (B cells) cells were likely present in the brains of RABV-62scFv treated mice over the course of the study<sup>180</sup>, the brains of these mice could only be harvested at the end of the experiment (28 days after viral inoculation). It is possible that by this timepoint, these cells may have migrated out of the brain and CNS. Together, these data indicated that ic inoculation was an effective delivery route to enable infection of the brain/CNS and that RABV-62scFv was apathogenic after ic administration.

Following on from the assessment of RABV-62scFv pathogenicity upon ic and iv infection, RABV-62scFv was taken forward to the second phase of *in vivo* experiments. Here, the applicability of RABV-62scFv for use as a post-exposure treatment was assessed. This study was designed to determine how effective ic RABV-62scFv treatment was in preventing mortality when given either simultaneously, three, or five days after lethal RABV infection. In this study RV437, an old-world lethal rabies virus isolated from a raccoon-dog, was used as the lethal challenge virus to infect mice im with a single dose into the thigh muscle of both hind legs. In total, five experimental groups were assigned for this study including two control groups (challenge-only and treatment-only) and three treatment groups (after lethal RV437 challenge on day 0, mice treated on either day 0, three, or five). As shown in Figure 5:4, mice treated on day 0 or three after im RV437 challenge displayed survival rates of 75% and 33% respectively. These results were highly significantly different (\*\*\*\*) from the challenge only group. The survival rate of mice treated at day five after lethal challenge was 0%. Together, these data indicate that RABV-62scFv was an effective method of post-exposure prophylaxis. However, the effectiveness of ic RABV-62scFv treatment exhibited a negative correlation with time after lethal challenge, with effectiveness reaching 0% at five days.

Throughout this study, mice were assigned a clinical score based upon pre-determined criteria (Table 5:3). Mice were scored twice daily and were terminated upon reaching clinical score three. One observation of mice treated with RABV-62scFv was that these mice remained symptomatic for longer than the mice in the challenge-only group. As shown in Figure 5:5, between the challenge-only group and the day three treatment group this difference was statistically significant (\*). Despite the average survival time after developing symptoms in the day 0 treatment group being longer than the day three treatment group, a statistical comparison was not possible due to a lack of data. In some groups, some mice were also seen to recover after developing score one symptoms. In summary, this suggests that even in cases where RABV-62scFv was unable to prevent the progression of symptoms, treatment was able to delay progression of disease.

Table 5:4 comprehensively illustrates serological titres of mice against RABV-62scFv and RV437 within the context of the progression of clinical scores after lethal challenge and treatment. As shown in Figure 5:4 and Figure 5:5, no mice within treatment-only groups developed symptoms and mice within challenge only group developed and progressed through clinical scores quickly. In the challenge only group, reciprocal antibody titres against RV437 ranged from 0 to 140 (average 62.7). These values were among the lowest titre values seen in responses against RV437 across all experimental groups. Reciprocal titre values against RABV-62scFv seen in the treatment only group ranged from 46.8 to 729 (average 336.3).

One third of mice simultaneously treated with RABV-62scFv and challenged with RV437 developed symptoms and after progressing to ethical endpoints were subsequently culled. However, of the nine remaining mice, one mouse (mouse 11) recovered after displaying symptoms of rabies for two days and another (mouse five) remained symptomatic but did not progress in clinical score for 10 days until the end of the experiment. In this experimental group, mouse 11 displayed high reciprocal titres of 814.6 and 420.9 for RABV-62scFv and RV437, respectively. These values were among the highest within this experimental group, potentially indicating how this mouse was able to recover from clinical symptoms. However, of most interest were the antibody titres seen within the sera of mouse five. This mouse exhibited titres approximately five times higher against both RABV-62scFv (4608) and RV437 (2187) than any other mouse within this experimental group. Ideally, this study would have been extended to observe whether mouse five and 11 would continue to display symptoms of rabies infection, recover, or begin to progress through clinical scores. Unfortunately, this was not possible. In cases where mice displayed high titres of anti-RABV antibodies (e.g. mouse 10

of the day 0 treatment group) yet still succumbed to infection, this may be explained by the generation of an anti-RABV immune response by the host, but this response being insufficient to control and clear infectious virus.

Similar findings to mouse 11 of the day 0 treatment group were found in the day three treatment group. Here, mouse four began to exhibit symptoms of rabies infection on day 16 after RV437 challenge and remained symptomatic for the remainder of the experiment. Mouse four also displayed high antibody titres against both RABV-62scFv and RV437. High antibody titres were also seen in mouse five and six, neither of which exhibited symptoms throughout the experiment.

As explored within Section 5.2.2.2, formalin-fixed paraffin embedded mouse carcasses underwent histopathological examination for the presence of lesions and RABV N protein in areas such as the cerebrum, hippocampus, thalamus, midbrain, cerebellum, brain stem, and spinal cord. With the exception of mouse six in the day 0 treatment group, RABV N protein presence in mouse sections followed expected results i.e., where assessed, mice that were culled due to symptoms of RABV infection were positive for N protein (and occasionally pathological lesions), while those that survived until the end of the experiment were not. It is important to note, however, that in these slides RABV N protein was visualised using the 5B12 (MyBioSource Inc., San Diego, CA, USA) antibody which was unable to distinguish between the N protein of RABV-62scFv and RV437. In the case of mouse six of the day 0 treatment group, both N protein and lesions could not be detected in any of the areas assessed. During this study, symptom onset in mouse six presented as weakness/dragging in both legs. Mouse six remained active throughout this period and was only culled due to an inability to reach food and water as the hind legs became increasingly weaker (while the upper limbs and mouse activity remained normal). Ideally, pathological lesion and RABV N protein presence analysis would have been performed on the quadricep muscles of this mouse. This may have given further insight into the cause of the symptoms displayed by mouse six, where RABV N protein or lesion presence in the quadricep muscles (and absence in the spine/brain) may explain the hind leg weakness/dragging observed. For example, the presence of pathological lesions in the quadriceps of this mouse may indicate that the paralysis observed was due to the damage caused by RABV-62scFv infection in the quadricep, however, this infection was controlled by the host immune response before widespread CNS and brain infection could occur. Unfortunately, while quadricep muscle sections were taken of all mice, non-specific background labelling prevented interpretation.

Together, the above discussions on the relationship between mouse survival, serological response, and symptomatic duration suggest the following conclusions may be drawn. When given concurrently with lethal ic RV437 challenge RABV-62scFv was, in the majority of cases, able to prevent the onset of disease in mice. In cases of symptom onset, RABV-62scFv was also able to clear symptomatic infection or at least delay the progression of symptoms. However, further research is needed to fully support these secondary claims. In the instance of concurrent RABV-62scFv treatment being able to clear symptomatic RABV infection, mouse 11 was only observed to have a hunched spine for two days before this symptom alleviated and the mouse allowed to progress until the end of the experiment. While hunched spine is a classic and well-known symptom of RABV infection in mouse models, no histopathological data exists to indicate that this symptom was due to RABV infection and not an extraneous factor. However, if the observations in mouse 11 were due to RV437 infection, the serological response data indicates that recovery was due to RABV-62scFv treatment. In the mice that progressed through clinical scores and were subsequently culled (mouse six and 10 in the day 0 treatment group), lower antibody titres against both RABV-62scFv and RV437 were seen. This is in contrast to mouse 11 which reported increased antibody titres. These discussions can be applied to the observations of mouse five in the day 0 treatment group. Where RV437 was cleared from mouse 11 resulting in the resolution of symptoms after two days, RV437 within mouse five was not cleared as easily (indicated by the length of the symptomatic period) and the prolonged exposure of RV437 to the host immune system resulted in a considerably increased antibody titre. As discussed previously, whether this markedly increased antibody titre would result in the resolution of symptoms is unknown.

When RABV-62scFv treatment was delayed until three days after lethal RV437 challenge, it was clear that similar effects to concurrent treatment were seen. However, due to the delay between challenge and treatment, the effect of RABV-62scFv treatment was clearly diminished. Alongside the survival rate decreasing from 75% to 33%, Figure 5:5 showed that these mice were symptomatic for, on average, a shorter period of time before progressing to clinical endpoints. By increasing the time between challenge and treatment, RV437 would be allowed to productively infect and replicate within the murine host before RABV-62scFv treatment was given. Therefore, when lethal challenge was treated concurrently with RABV-62scFv, a combination of the early presence of 62-71-3 scFv within the brain (either from replicating RABV-62scFv or included within the initial inoculate) and stimulation of the host immune response was sufficient to clear RV437. This may explain why the majority of day 0 treatment mice which did not show symptoms of viral infection exhibited relatively low levels

of neutralising antibody against both RABV-62scFv and RV437. In contrast, due to the delay between challenge and treatment, an early immune response and presence of 62-71-3 scFv in the brain would not be sufficient for RV437 clearance. A more rigorous immune response was required, resulting in a greater antibody response. Therefore, in cases where mice progressed through clinical scores and were culled, this may be because a sufficient immune response was not generated in time. This supports the observations seen in the day five treatment group, where regardless of serological response mice quickly progressed through clinical scores and were subsequently culled. Of interest of the day five treatment group were mice four, six, and 10, which were symptomatic for approximately seven days (other mice within this experimental group were symptomatic for approximately one day). While the serological data for mouse six supports previous observations that increased antibody titres correlate with a delay in symptom progression, this is not supported by the serological data from mouse 10, and data from mouse four was not available.

One limitation to the study investigating the effectiveness of RABV-62scFv as a post-exposure treatment was the method used to lethally challenge mice with RV437. In previous studies, lethal challenge with RV437 was comprised of only a single injection of RV437 into the hind leg of mice. However, this has previously resulted in the occasional survival of mice. Therefore, in an effort to reduce the numbers of mice used in this experiment in keeping with ethical guidelines it was decided that two doses (one in each leg) would be used. While this was effective in producing 100% mortality of the challenge only group, it may have had undesirable effects on the treatment groups. In previous studies, mice that survived single-leg RV437 challenge have been reported to show weakness/dragging only in the inoculated leg (Internal APHA communication, David Selden). This suggests that, in these cases, effects such as single-leg paralysis/weakness caused by RABV infection may persist even after viral clearance. Therefore, when scoring mice according to the conditions described in Table 5:3 mice may have been culled prematurely. For example, if mice were single leg challenged within this study, after RABV-62scFv was able to clear infection, mice may have been left with a (semi) paralysed leg but would otherwise not fit the characteristics required for culling. However, in this study, regardless of whether mice may have cleared RV437 infection, paralysis in both legs would have reached the requirements for the humane endpoint (inability to reach food/water) and required subsequent culling. Interestingly, this may have been the case in some mice where, aside from loss of function in one/both legs, mice appeared healthy.

## Chapter 6 - DISCUSSION AND FUTURE WORK

This thesis describes the design, cloning, viral rescue, and *in vivo* assessment of a novel therapeutic for the treatment of clinical rabies based upon the expression of a fluorescent neutralising scFv molecule from a live attenuated RABV vector.

The first results chapter (Chapter 3) of this thesis focused on the cloning and rescue of the RABV-62scFv vector. In short, RABV-62scFv was comprised of the attenuated RABV backbone vector RABV-cSN encoding the gene for a secreted 62-71-3 scFv molecule. A K226R mutation was introduced in the RABV G gene to prevent self-neutralisation by the expressed 62-71-3 scFv molecule. One of the first decisions made was to insert the 62-71-3 scFv expression cassette in 'position 1' of the RABV genome (upstream of the N gene). This site was chosen due to the transcriptional gradient present during RABV replication which would result in large amounts of scFv being produced. Furthermore, as has been explored in other publications, first position gene insertion is also an effective method of viral attenuation<sup>334</sup>. While RABV-cSN is traditionally regarded as an attenuated RABV strain, it is still pathogenic when delivered ic. Therefore, the additional contribution to attenuation arising from the first position insertion may explain why RABV-62scFv was apathogenic when administered ic (Chapter 5). While this attenuation was beneficial, it may have had detrimental effects on viral replication and dissemination. Chapter 4 explored, amongst other aspects, the growth kinetics and viral dissemination of RABV-62scFv in cell culture. When comparing the growth kinetics of RABV-cSN and RABV-mCH, it was clear that the insertion of an additional transcriptional unit upstream of the N gene caused decreased growth kinetics. This observation was also present in the results from the viral dissemination assay (Section 4.2.3). Together (and consistent with observations seen in VSV studies<sup>334</sup>), these results indicated that the attenuation seen in the *in vivo* studies was due to a decreased ability to infect or replicate within cells. While this thesis has not sought to distinguish between the ability of RABV-62scFv to infect and replicate within cells, it is likely that the incorporation of an additional ORF upstream of the N gene attenuated RABV-62scFv by the decreased expression of downstream genes and not by interfering with the ability of RABV-62scFv to infect cells. The efficiency of cellular infection is largely independent of gene expression profiles, as infection is solely reliant on the binding of the RABV G protein to a cellular receptor. However, it has been thought that some pathogenic RABV strains restrict G gene expression, resulting in the decreased incorporation of G protein into RABV particles<sup>197</sup>. While this is performed predominantly to evade the immune system, decreased incorporation of G protein into RABV particles may also result in less efficient cellular infection. While apathogenicity is an essential aspect for any effective LAVV-based

treatment, a decreased ability to infect or replicate within cells means that less cells are infected after RABV-62scFv inoculation, and therefore less cells are able to produce 62-71-3 scFv. To address this issue, three avenues exist.

The first and most simple avenue would be to administer mice with a higher concentration of virus. As shown in Figure 5:1, only four concentrations of RABV-62scFv were assessed during the *in vivo* study assessing toxicity ( $10^1$ - $10^4$  FFU/dose). To determine the highest dose that could be administered to mice *ic*, while still maintaining safety, concentrations up to  $10^8$  FFU could be assessed. Similar concentrations have been used previously while assessing apathogenicity of attenuated RABV vectors in mice <sup>163</sup>, and higher inoculum concentrations could result in a greater number of infected cells. Secondly, instead of inserting the 62-71-3 scFv expression cassette prior to the N gene, the insertion site could be moved downstream. As described elsewhere <sup>180</sup>, in the majority of studies where NVEs have been inserted into a RABV genome, this insertion occurs between the G and L genes. If the cause of attenuation was due to the lower amounts of downstream proteins produced, expression of N, P, M, and G protein will be conserved by placing the scFv cassette between the G and L genes. However, this change of insertion site would also be accompanied by a decreased production of 62-71-3 scFv and the potential abolition of the attenuation gained by inserting the 62-71-3 scFv cassette in the first position of the genome. While the increased cellular infection and replication may counter the effects of decreased 62-71-3 scFv production, insertion of the 62-71-3 scFv expression cassette in a different viral vector may address pathogenicity concerns when delivered *ic*. Furthermore, an increased rate of replication will increase the risk of reversion to pathogenicity with the associated likelihood of mutations occurring. Lastly, if it was desired that the attenuated viral vector be RABV-based, use of RABV vectors containing aa mutations at positions 333 and 194 (alongside possible duplication of mutated G proteins) may be a viable alternative <sup>228,235-239</sup> (other attenuated viral vectors are discussed in greater detail in Section 1.6.1.1).

Another alternative would be to use VSV as the expression vector for 62-71-3 scFv. VSV has been thoroughly researched as a platform for LAVVs and in some cases has been approved for use in humans <sup>371,372</sup>. Of particular interest, studies utilising glioblastoma-targeting VSVs have demonstrated that even with first position NVE insertions and *iv* administration, these vectors are able to quickly infect target cells within days of administration <sup>332,334</sup>. Using VSV as the vector would aid in alleviating issues in sufficient cell infection for scFv production, in addition to concerns surrounding viral reversion to pathogenicity. Furthermore, while the *iv* route has been shown to be ineffective for the delivery of RABV-62scFv, this route may be a viable alternative for VSV delivery.

During the process of cloning the 62-71-3 scFv expression cassette into RABV-cSN, both the E-tag and 8xHis tag were removed from the region between the 62-71-3 scFv and the mCHERRY fluorophore. This was performed to prevent cell death during virus rescue because, as discussed previously, this may have arisen only due to the E-tag. However, this requires further study<sup>342-347</sup>. Regardless, the presence of a labelling or purification tag (such as a 8xHis tag) would greatly simplify vp62-71-3 scFv identification, purification, and quantitation.

Section 4.2.4 explores the production of vp62-71-3 scFv over time by ELISA. However, due to the lack of an appropriate standard control of known concentration, results are only displayed as relative OD450 units. In previous research which studied the initial expression of 62-71-3 as an scFv, *N. benthamiana* plants were used. While stocks of plant-produced 62-71-3 scFv were available, these contained both an E-tag and a DsRED fluorophore in place of mCHERRY. This made the use of plant-produced 62-71-3 scFv as a standard control within the ELISA unsuitable for two reasons. First, the effect of the E-tag/8xHis tag and DsRED replacement on the ability of 62-71-3 scFv to bind RABV G protein was unknown. Secondly, the ELISA employed in Section 4.2.4 relied on the use of a Rat anti-mCHERRY (5F8, chromotek) antibody to bind vp62-71-3 scFv bound to the pre-coated Platelia® Rabies II Kit (BioRad) ELISA plate. While the anti-mCHERRY antibody was claimed to strongly bind multiple red fluorescent proteins including DsRed, mRFP, mPlum, mRFPruby, mScarlet, and tdTomato by the manufacturer (chromotek) it was unknown how the binding of DsRED vs mCHERRY would affect the results of the ELISA. Monomeric mCHERRY could also not be used to quantitate vp62-71-3 scFv in cellular supernatant, as even if a monoclonal anti-mCHERRY antibody was used to coat the ELISA plate and a HRP-conjugated polyclonal anti-mCHERRY antibody used to bind the scFv-linked mCHERRY, this would not be able to distinguish between full-length vp62-71-3 scFv mCHERRY and the monomeric mCHERRY seen in Figure 4:6.

To effectively quantitate the amount of vp62-71-3 scFv produced over time after cellular infection, two avenues are available. If a tag such as a 8xHis tag was included within the vp62-71-3 scFv polyprotein, monomeric mCHERRY conjugated to the same tag would represent a suitable ELISA standard control as both an anti-mCHERRY and anti-tag antibody could be used. However, this would not consider the ability of the quantified vp62-71-3 scFv to bind RABV G, and any post-translation cleavage of vp62-71-3 scFv which resulted in the production of a tag-mCHERRY fusion protein would be indistinguishable from full-length vp62-71-3 scFv by ELISA. However, by including a purification tag within the vp62-71-3 scFv polyprotein, vp62-71-3 scFv could be effectively purified from cell culture supernatant. For example, a nickel affinity column could be used to purify His-tagged proteins from the cell-culture supernatant, and size

exclusion chromatography could be used to purify full-length 62-71-3 scFv. Densitometry could then be performed on a western blot (visualised with an anti-8xHis antibody) using a 8xHis-tagged protein of known concentration to create a standard curve and identify the concentration of the appropriate full-length vp62-71-3 scFv band.

Once a stock of full-length vp62-71-3 scFv of known concentration was available, this could be used as a standard control within the ELISA used in this project to quantitatively measure vp62-71-3 scFv production over time. This approach would satisfy the two caveats associated with the use of 8xHis tagged mCHERRY to quantify vp62-71-3 scFv production by ELISA. Furthermore, with a method for the purification and quantification of vp62-71-3 scFv within cell culture supernatant, neutralisation assays could be performed investigating the relationship between the concentration of vp62-71-3 scFv and ability to neutralise viruses including RV437 and CVS-11.

The antibody 62-71-3 has been shown to target AS-I of the RABV G protein with epitope mapping revealing specificity to aa residue K226<sup>302,304,305</sup>. Furthermore, 62-71-3 has also been shown to be effective at neutralising a wide range of phylogroup I Lyssaviruses<sup>302,307</sup>. While these characteristics support the selection of 62-71-3 scFv within the viral vector, recent research has indicated that some alternatives may provide a better avenue for future investigation. These avenues include the incorporation of a second RABV-neutralising antibody (either within the same viral vector or delivered alongside in a RABV-neutralising antibody expressing virus cocktail) or the addition of an Fc region to the neutralising scFvs. The principle behind the incorporation of a second RABV-neutralising antibody is similar to why some monoclonal antibody therapies exist as a combination of two monoclonal antibodies<sup>373,374</sup>. In cases where one of the antibodies are unable to neutralise a virus stain, or an escape mutant is generated through selection pressure, it may be neutralised by the other<sup>375</sup>. Studies where multiple G genes have been inserted into RABV has demonstrated RABV has a maximum coding capacity of at least 18 kb<sup>228,235-239</sup>. Therefore, inserting an additional scFv into the RABV backbone could be easily tolerated and would result in the expression of a cocktail of antibodies from a single cell. However, inserting a second ORF into the RABV genome may further decrease the production of all downstream genes meaning one antibody is always be produced in greater amounts than the other. One alternative is to deliver the post-symptom onset therapy as a cocktail of viruses with each virus expressing a single RABV-neutralising antibody. However, each expressed antibody must be highly characterised. In the case of 62-71-3, research has demonstrated that inducing a K226R mutation in the RABV G gene drastically reduces the ability of 62-71-3 scFv to bind. If any further neutralising antibody

sequences were incorporated within RABV vectors, similar binding-reducing mutations would have to be incorporated to prevent self-neutralisation.

Recently, a study by de Melo et al <sup>310</sup>, demonstrated that mice could be cured of symptomatic rabies infection if a combination of two human monoclonal antibodies were delivered by intracerebroventricular infusion. A part of this study investigated the necessity of the Fc region of these antibodies. Here, a Leu-alanine (Ala)-Leu-Ala mutation (Leu234Ala, Leu235Ala) was introduced into the Fc region of the RVC20/RVC58 antibodies assessed. This mutation has been demonstrated to prevent binding of Fc regions to FcγRI/IIa/IIIa and activate complement for IgG1/4 molecules, leading to less potent T cell activation and cytokine release <sup>376,377</sup>. The investigation conducted by de Melo et al <sup>310</sup> revealed that the Fc region played a vital role in the clearance of RABV when treatment was started late in infection as while these antibodies retained their ability to effectively neutralise multiple RABV strains *in vitro* they performed significantly worse when used to clear RABV infection within mice. In this project, it was decided to express scFv molecules from a RABV backbone due to their small (protein and coding sequence) size, enhanced tissue penetration, neutralising ability, and use in previous research <sup>302,307</sup>. However, scFv molecules lack an Fc region. Dias de Melo's study <sup>310</sup> indicates that an Fc region is vital for effective RABV clearance from the brains of mice and, therefore, future studies investigating the use of a virally expressed neutralising protein should further investigate the inclusion of an Fc region. While this would make scFv molecules unsuitable, a scFv-Fc fusion protein would retain many of the characteristics of an scFv molecule while allowing Fc receptor interactions <sup>378,379</sup>.

The second results chapter of this thesis focused on the identification and characterisation of both the vp62-71-3 scFv protein and RABV-62scFv. The effect of RABV-62scFv infection on cell viability was assessed, alongside RABV-62scFvs growth kinetics and dissemination between cells. Alongside confirming the identity of the neutralising protein present in the supernatant of RABV-62scFv infected cells, vp62-71-3 scFv expression over time was assessed and the ability of vp62-71-3 scFv to both bind RABV G protein and neutralise infectious virus was investigated. Lastly, the ability of RABV-62scFv to cross an *in vitro* BBB model was also examined as an indication to its applicability as an iv treatment.

As discussed previously, the K226R mutation was included in the G gene of RABV-62scFv to minimise self-neutralisation by the expressed 62-71-3 scFv. This was based upon previous data from Both et al <sup>302</sup> which showed that the K226R mutation alone reduced binding of 62-71-3 to the RABV G protein by approximately 75%. From this research, two questions remain: Firstly, is it possible to further reduce the residual binding seen after the inclusion of the K226R mutation; and secondly, is the residual binding seen *in vitro* of importance *in vivo*. In the study

conducted by Both et al <sup>302</sup>, two AS-I mutations were identified and assessed for effect on 62-71-3 scFv binding: K226R and Gly229Glu. Both et al <sup>302</sup> demonstrated that the K226R mutation reduced binding by 75%, while the Gly229Glu mutation reduced binding by 60%. In this thesis, only the K226R mutation was included within the RABV G gene of RABV-62scFv as this was the dominant mutation involved in reducing 62-71-3 scFv binding. Furthermore, producing a functional RABV-cSN G protein containing both the K226R and Gly229Glu mutation had not been attempted previously. In an effort to further reduce residual 62-71-3 scFv binding, this pseudotype neutralisation assay should be repeated with glycoproteins receiving both the K226R and Gly229Glu mutations. If the incorporation of both these mutations into a single glycoprotein was effective in further reducing the binding of 62-71-3, both these mutations should be introduced into the G protein of RABV-62scFv.

In the study by Both et al <sup>302</sup>, the effect of the K226R and Gly229Glu mutations on the ability of 62-71-3 to neutralise RABV G protein was only assessed in pseudotype viruses, not live virus. In this thesis, during the process of rescuing different variations of RABV-62scFv it was discovered that the K226R mutation was an absolute necessity for successful virus rescue. However, the effect of residual 62-71-3 scFv binding on RABV-62scFv was not investigated. To assess whether vp62-71-3 scFv is able to neutralise RABV-62scFv a neutralisation assay could be performed against RABV-mCH-K226R. Using RABV-mCH-K226R is ideal as unlike RABV-62scFv there is no vp62-71-3 scFv present in the supernatant of infected cells and therefore any effects of vp62-71-3 mediated neutralisation would be immediately apparent. As discussed previously, one explanation regarding the characteristics of RABV-62scFv seen in Chapter 4 (such as slow cellular dissemination and reduced growth kinetics) is that this was due to the effects of residual vp62-71-3 scFv binding to RABV-62scFv inducing less efficient cell binding, internalisation, and uncoating within endocytic vesicles. Secreted vp62-71-3 scFv may also bind RABV G proteins present on the surface of RABV-62scFv infected cells (discussed in more detail in Section 4.3). However, the observations of slow cellular dissemination and reduced growth kinetics are also present in viruses such as RABV-mCH and RABV-mCH-K226R. This indicates that it was not the residual binding of 62-71-3 scFv to the RABV-62scFv virion (or to the surface of infected cells) but the insertion of an additional ORF upstream of the N gene.

The final experiment of Chapter 4 focused on the use of an *in vitro* BBB model to assess the applicability of RABV-62scFv as an iv treatment *in vivo* (Section 4.2.8). After culturing hCMEC/D3 cells on a semi-permeable tissue culture membrane transwell insert, LY was used to assess membrane permeability daily. In further experiments, the ability of RABV-62scFv to infect the hCMEC/D3 monolayer and produce virions was assessed. RABV-62scFv was allowed to infect the hCMEC/D3 monolayer, alongside heat-inactivated RABV-62scFv samples, for one

h before cells were washed and samples taken 72 h later. While this experiment demonstrated the ability of RABV-62scFv to cross the *in vitro* hCMEC/D3 BBB model, there were some questions which were not addressed. Firstly, hCMEC/D3 integrity was only assessed in the first phase of experiments when attempting to determine when a monolayer of appropriate integrity was formed. However, it was not investigated whether this monolayer-maintained integrity over the course of viral infection. It was possible that due to the cellular infection observed in Figure 4:11 that monolayer integrity decreased and RABV-62scFv virions were allowed to pass through to the basolateral compartment. This may explain the presence of RABV-62scFv RNA (in the live-virus treated groups) within the basolateral compartment after 72 h. Assessment of monolayer integrity during and after RABV-62scFv infection and treatment with heat-inactivated RABV-62scFv was initially attempted, however, these experiments were unsuccessful. Due to the high containment regulations surrounding work with live RABV, once hCMEC/D3 monolayers were infected with RABV-62scFv they could not be removed from high containment facilities. Unfortunately, a suitable instrument for reading LY fluorescence (excitation peak at 428 nm and an emission peak at 536 nm) was not available and the only instrument accessible was a GloMax-Multi Detection System (Promega) where the most appropriate fluorescence module available was only able to excite at 490 nm and measure emission at 510-570 nm. Apical and basolateral samples could not be removed from high containment and current sample removal procedures from high containment required BPL treatment and a 10-day wait period. As discussed, within the current setting it was not possible to measure hCMEC/D3 permeability after viral infection, however, this could be easily achieved with the purchase of the appropriate equipment and subsequent transport into the high containment facility.

The hCMEC/D3 cell line was chosen for use as a BBB model due to its ease of use, thorough characterisation, and usage in multiple areas of research<sup>380</sup>. However, there are multiple alternatives to culturing the hCMEC/D3 cell line on a porous cell culture insert and these have been reviewed thoroughly elsewhere<sup>381-383</sup>. One alternative to the hCMEC/D3 cell line used in this thesis are dynamic BBB models. Compared to the culture of a hCMEC/D3 monolayer on a motionless cell-culture insert, dynamic BBB models utilise fluid pumps to emulate the effect of blood flow and contain cultures of multiple cell types. These fluid pumps produce shear stress conditions similar to *in vivo* environments to increase TJ protein ZO-1 expression. Furthermore, on the luminal and abluminal sides of the gas-permeable tubing comprising the system (which allow O<sub>2</sub> and CO<sub>2</sub> exchange), bone marrow microvascular endothelial cells and astrocytes are cultured, respectively. Together, these differences result in a BBB model much more relevant to *in vivo* conditions than hCMEC/D3 transwell alternatives. Dynamic models are, however,

also associated with various disadvantages such as significant equipment cost (such as viable speed pumps and gas permeable tubing) and required operator skill <sup>381-384</sup>. Due to the reasons discussed above, if a dynamic flow system was used as a BBB model in place of the hCMEC/D3 monolayer culture, this may have given results which indicated that RABV-62scFv was unsuitable for iv treatment.

The final results chapter of this thesis focused on the *in vivo* assessment of RABV-62scFv as a post-symptom onset treatment. This was to be investigated in three distinct stages; first, by which routes is RABV-62scFv non-toxic (or does not elicit symptoms of RABV infection) in a mouse model; second, is RABV-62scFv able to prevent the onset of disease if given simultaneously or after lethal RABV infection (but before the onset of symptoms); and lastly, is RABV-62scFv able to prevent mortality if given after symptom onset from lethal RABV challenge.

In the first stage of *in vivo* assessment, four concentrations ( $10^{1-4}$  FFU/Dose) of RABV-62scFv were assessed by either the ic or iv route. The ic route was selected as this represented the fastest route of delivering RABV-62scFv directly into the brain where it may infect local cells and produce 62-71-3 scFv. While the iv route is unconventional for infecting *in vivo* models with RABV, it represented a delivery route easily performed in humans, in contrast to an ic delivery of virus. As described in Section 5.2.1, delivery of RABV-62scFv by the ic route was effective in promoting a strong antibody response associated with clearance of RABV (both N protein and live virus) from the brain. Similarly, an antibody response was seen against RABV-62scFv delivered by the iv route. However, the failure of the pathogenic control virus (RABV-CVS-mCH) to elicit infection and symptom onset after iv administration suggests that iv administration did not result in virus infection and was, therefore, unsuitable as a route of inoculation. In contrast, RABV-CVS-mCH delivered ic gave expected results with mortality increasing with inoculum concentration. Mice that were culled due to the onset of symptoms were also FAT and RTCIT positive and histopathological assessment of a representative mice revealed the presence of RABV N protein (sporadically associated with CD3<sup>+</sup> cells) within the midbrain, hippocampus, and thalamus regions of the brain.

In summary, this study demonstrated the RABV-62scFv was apathogenic when delivered by the ic route (which itself was shown to be an effective method of delivery of RABV directly to the brain) and resulted in the generation of a strong immune response, here characterised by the generation of neutralising antibodies, which allowed clearance of RABV-62scFv from the brain. This study would strongly benefit from a more in-depth investigation into areas such as maximum doses of RABV-62scFv and the immune response underlying the clearance of RABV-62scFv from the brains of infected mice. Maximum doses of RABV-62scFv could be assessed by

inoculating mice with higher doses of RABV-62scFv until pathogenic effects are seen. As discussed previously, equivalent studies have shown that ic doses up to  $10^7$  FFU may be apathogenic<sup>163</sup>.

An in-depth investigation into the mechanisms of RABV-62scFv clearance from the brains of infected mice was not possible in this study as mice were only culled either upon displaying symptoms of infection or at the end of the experiment. This resulted in a disparity between mice in the pathogenic control group and experimental RABV-62scFv treatment groups. For example, when trying to compare histopathological or FAT/RTCIT results between a mouse given RABV-CVS-mCH and RABV-62scFv, while the mouse given RABV-CVS-mCH may be culled on day eight, the mouse given RABV-62scFv would be culled at the end of the experiment (day 28). This 20-day difference may mean that any immune cells of interest, which equivalent research<sup>180</sup> indicates should be present and are likely the mediators of RABV-62scFv clearance, may have migrated out of the brain. This explains the absence of CD79a/CD3/MAC387 positive cells seen in the brains/CNS of RABV-62scFv challenged mice. This delay also caused complications when attempting to assess cytokine/chemokine upregulation in the brains of mice. RNA extraction, cDNA synthesis, and qPCR analysis of IFN $\gamma$ , ISG15, MX dynamin-like GTPase 1 (Mx1), IFN-1 $\beta$ , IL-6, CCL5, and CxCL10 genes was attempted to gain a greater understanding of the mechanisms behind the clearance of RABV from the brains of mice. However, the limitation of culling RABV-62scFv mice at the end of the experiment meant that mRNA levels had returned to similar levels to virally unchallenged mice and this was unlikely to be a true reflection of the transcriptome present during the clearance of RABV-62scFv from the brains of mice. The shortcomings discussed above may be addressed by the design and execution of an additional *in vivo* mouse experiment. Here, mice would be challenged ic with  $10^4$  FFU of RABV-62scFv and culled daily starting from day 0. Through histopathological, serological, and cytokine/chemokine mRNA analysis, the upregulation/downregulation of various chemokines, infiltration of immune cells (such as T cells, B cells, and APCs), and serological antibody response could be assessed. Furthermore, compounds such as sodium fluorescein could be used to assess changes in permeability of the BBB over infection<sup>89</sup>.

After establishing that RABV-62scFv did not result in mortality, the ability of ic RABV-62scFv treatment to prevent mortality if given post-infection (but prior to symptom onset) was assessed. This involved challenging mice with two im doses of RV437 accompanied by ic treatment with RABV-62scFv at either 0, three, or five days post-challenge. As described in Section 5.2.2 and shown in Figure 5:4, RABV-62scFv treatment was effective in preventing mortality (or the progression of symptoms to ethical endpoints) in 75% of mice treated concurrently with lethal challenge, and 33% of mice when treated three days after lethal

challenge. When mice were treated with RABV-62scFv five days after lethal challenge with RV437, no mice survived until the end of the experiment. While this experiment satisfied the query of whether ic RABV-62scFv treatment could prevent symptom onset/progression, there are multiple areas which, if this study was repeated, could be improved upon. Within this study, mice were challenged with two im doses of RV437. However, as discussed, this may have resulted in mice being prematurely culled. In a future study, only a single dose of challenge virus (such as RV437) would be used although this may mean that more mice are needed to attain sufficient statistical power. Furthermore, as some mice continued to display symptoms until the end of the experiment, this study could have been extended to determine whether these mice would eventually succumb to infection, symptoms would resolve, or remain symptomatic. If mice were to remain symptomatic (i.e. signs of limping or paralysis), one explanation may be that while RV437 had been cleared from local muscle and nervous tissues, damage to these areas had already occurred and could not be reversed by the clearance of RV437. This study effectively demonstrated that as time after lethal viral challenge increased the effectiveness of RABV-62scFv treatment decreased. This may be due to multiple reasons. Firstly, as discussed previously, due to the design of the first *in vivo* experiment (Section 5.2.2) no data were available on the ability of RABV-62scFv to infect the brains of mice after administration. Therefore, the increase in survival in mice treated with RABV-62scFv may be due to the ic inoculation of an attenuated RABV (and not necessarily the production of 62-71-3 scFv from infected cells), an event known to elicit an antibody response and prevent mortality in lethally challenged mice<sup>180</sup>. An appropriate control which should be included in further experiments is a virus such as RABV-mCH-K226R. This virus is identical to RABV-62scFv but without the scFv gene, therefore, any effects due to the production of 62-71-3 scFv within the brain would result in a significantly increased survival rate.

In summary, the *in vivo* experiments within this thesis leave the following questions unanswered: How effective is RABV-62scFv at infecting cells within the brain, how quickly after ic RABV-62scFv administration does 62-71-3 scFv production and BBB permeabilisation occur, and does inclusion of 62-71-3 scFv within the RABV-62scFv vector have a significant effect on mouse survival if given as a PIT. While further *in vivo* experiments using RABV-62scFv may provide answers to these questions, it may be more appropriate use a different viral vector. VSV is a rhabdovirus and has been extensively used in research for the treatment of brain cancers due to its fast replication cycle (approximately three h which is associated with significant cell death) and ability to specifically infect a large variety of tumour cell lines<sup>385-389</sup>. For use in the context of this thesis, pseudotyping the VSV G protein with a RABV G protein would allow VSV to utilise the same cellular receptors as RABV and infect the same population

of cells. Pseudotyping VSV with RABV G gene is a frequently used technique in neuronal circuitry tracing research <sup>390</sup>. VSV has also been shown to infect brain tumour cells whether administered ic <sup>391-393</sup> or iv <sup>332,334,394,395</sup>. While the success of iv-delivered VSV may be due to the increased permeability of the blood-tumour barrier compared to the BBB <sup>395,396</sup>, ic administration has also been shown to be an effective method of VSV delivery into the brain <sup>391-393</sup>. However, while iv delivery may be apathogenic <sup>397,398</sup>, ic delivery (even using attenuated VSV strains) is often pathogenic. To circumvent this pathogenicity, success has been seen in mouse models which were 'primed' iv with VSV 18 h before ic inoculation of attenuated VSV strains <sup>399</sup>. In the case of using a RABV G gene pseudotyped VSV vector expressing 62-71-3 scFv, while mouse models may be primed with RABV G pseudotyped VSV similar to previous research, there is potential that the exposure to wild type RABV (which is causing the disease) may act in a similar way to the priming, which means RABV G gene pseudotyped VSV may be administered immediately.

In conclusion, this thesis has focussed on the development of an attenuated RABV which expresses the scFv 62-71-3. This scFv was secreted out of the host cell upon production, was fluorescent under UV illumination due to the presence of an mCHERRY fluorophore and was highly neutralising against RABV. The RABV vector also included a K226R site mutation in the G gene to prevent self-neutralisation by the produced scFv. After successful cloning and rescue of this virus (named RABV-62scFv), it was taken forward to *in vitro* assessment. Here, cellular toxicity, growth kinetics, cell-to-cell viral dissemination, and 62-71-3 scFv production kinetics was assessed. RABV-62scFv was then taken forward to *in vivo* studies where ic administration was shown to be apathogenic to mice and could significantly increase survival if given either concurrently or three days after lethal im RABV challenge. While there are some avenues of research which require a more thorough investigation (discussed above) the investigations within this thesis support the mechanism of a virally expressed neutralising protein for the treatment of clinical RABV infection.

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## Appendix

### Appendix 1 - STANDARD BUFFERS AND SOLUTIONS

|                                   |                                                                                                                                                                                                                                                                                                                                                                                                                                                                              |
|-----------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>0.1 M PBS<br/>(Sorensen's)</b> | <p>0.1 M PBS (Sorensen's) was prepared on-site by Animal and Plant Health Agency Biological Products Unit.</p> <p><u>Solution A (one litre)</u></p> <p>2.9 g Disodium Hydrogen Phosphate</p> <p>4.3 g Sodium Chloride</p> <p><u>Solution B (one litre)</u></p> <p>4.5 g Potassium Dihydrogen Orthophosphate</p> <p>4.3 g Sodium Chloride</p> <p>Combine solution A and B (870 mL and 130 mL, respectively) and pH adjusted to 7.4 before autoclaving at 121°C for 15 min</p> |
| <b>LB Agar</b>                    | <p>LB agar was prepared on-site by Animal and Plant Health Agency Biological Products Unit.</p> <p>7.5 g Tryptone</p> <p>2.5 g Sodium Chloride</p> <p>2.5 g Yeast Extract</p>                                                                                                                                                                                                                                                                                                |

|                   |                                                                                                                                                                                                                                                                                                                                       |
|-------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
|                   | <p>7.5 g of Agar</p> <p>Add distilled water to a final volume of one litre. pH then adjusted to 7.5 using 1 M Sodium Hydroxide before autoclaving at 121°C for 15 min.</p>                                                                                                                                                            |
| <b>LB Broth</b>   | <p>LB Broth was prepared on-site by Animal and Plant Health Agency Biological Products Unit.</p> <p>7.5 g Tryptone</p> <p>2.5 g Sodium Chloride</p> <p>2.5 g Yeast Extract</p> <p>Add distilled water to a final volume of one litre. pH then adjusted to 7.5 using 1 M Sodium Hydroxide before autoclaving at 121 °C for 15 min.</p> |
| <b>Ampicillin</b> | Ampicillin (Sigma) was stored at -20 °C upon delivery.                                                                                                                                                                                                                                                                                |
| <b>Kanamycin</b>  | Kanamycin (Sigma) was stored at -20 °C upon delivery.                                                                                                                                                                                                                                                                                 |
| <b>ATV</b>        | <p>ATV was prepared on-site by Animal and Plant Health Agency Biological Products Unit.</p> <p><u>One litre of 10x Trypsin Versene</u></p> <p>80 g sodium chloride</p> <p>4 g potassium chloride</p> <p>10 g glucose</p>                                                                                                              |

|                                                     |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |
|-----------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
|                                                     | <p>5.8 g sodium hydrogen carbonate</p> <p>2 g Versene</p> <p>10 mL of phenol red</p> <p>Distilled water added to a final volume of one litre.</p> <p>After mixing, trypsin was added at a 1:250 dilution and solution sterilised through a 0.2µm filter.</p> <p><u>1x ATV solution</u></p> <p>50 mL of 10x ATV added to 445 mL of sterilised water</p> <p>5 mL of Gibco™ Penicillin-Streptomycin (10,000 U/mL).</p> <p>10x Trypsin Versene was stored at -20°C, while 1x ATV was stored at 4°C.</p> |
| <b>TAE Buffer<br/>(Tris-acetate<br/>EDTA) (50X)</b> | <p>242 g Tris base</p> <p>57.1 mL Glacial acetic acid</p> <p>100 mL 0.5M EDTA (pH 8)</p> <p>Distilled water to one litre and autoclave to sterilise.</p>                                                                                                                                                                                                                                                                                                                                            |
| <b>TE Buffer (Tris-<br/>EDTA)</b>                   | <p>10 mM Tris-HCL (pH 8.0)</p> <p>1mM EDTA</p>                                                                                                                                                                                                                                                                                                                                                                                                                                                      |
| <b>Lucifer Yellow<br/>Transfer buffer</b>           | <p>25mM Tris</p> <p>190mM glycine</p> <p>20% methanol</p>                                                                                                                                                                                                                                                                                                                                                                                                                                           |

|                                         |                                                                                                                                                                                                                                                                                                                                                                                                                                                                |
|-----------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>Lucifer Yellow CH dilithium salt</b> | Lucifer Yellow CH dilithium salt was purchased from Merck (L0259). Upon receipt, Lucifer yellow was stored at 4 °C. For use, Lucifer yellow was resuspended in water and stored at 4 °C, shielded from light.                                                                                                                                                                                                                                                  |
| <b>Lucifer Yellow Transport Medium</b>  | Lucifer yellow transport medium used in the hCMEC/D3 blood-brain barrier study was 25 mM HEPES, 145 mM NaCl, 3 mM KCl, 1 mM CaCl <sub>2</sub> , 0.5 mM MgCl <sub>2</sub> , 1 mM NaH <sub>2</sub> PO <sub>4</sub> , 5 mM glucose at pH 7.4 <sup>321</sup> .                                                                                                                                                                                                     |
| <b>TBS- 0.1% Tween 20</b>               | 20mM Tris (pH 7.5)<br><br>150mM NaCl<br><br>0.2% Tween 20                                                                                                                                                                                                                                                                                                                                                                                                      |
| <b>Blocking buffer</b>                  | 100ml TBS-Tween 20<br><br>5% Marvel milk powder                                                                                                                                                                                                                                                                                                                                                                                                                |
| <b>NuPAGE™ MOPS SDS Running Buffer</b>  | NuPAGE™ MOPS SDS Running Buffer was purchased from Invitrogen (NP0001) and resuspended to 1x in sterile H <sub>2</sub> O.                                                                                                                                                                                                                                                                                                                                      |
| <b>0.3% Agarose-GMEM overlay media</b>  | 12.5 g Glasgow Minimum Essential Medium (Merck, G6148) powder<br><br>3 g of UltraPure™ Low Melting Point Agarose (Invitrogen, 16520100)<br><br>Made up to 1 L with sterile water<br><br><br><br>Briefly, GMEM powder was combined with 970 mL of sterile water and then warmed to 37 °C. 3 g of agarose was then heated with 30 mL of sterile water until completely dissolved. GMEM media and agarose solution was then thoroughly mixed and stored at 37 °C. |

|                                                     |                                                                                                                                                                                                                                                          |
|-----------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>SYBR™ Safe DNA Gel Stain</b>                     | SYBR™ Safe DNA Gel Stain was purchased from Invitrogen (S33102) and was stored at room temperature.                                                                                                                                                      |
| <b>SOC Media</b>                                    | SOC media was supplied with all orders of NEB® 5-alpha Competent <i>E. coli</i> (C2987H) and NEB® Turbo Competent <i>E. coli</i> (C2984H). SOC media was stored at room temperature.                                                                     |
| <b>Glasgow's Modified Eagle Medium (GMEM)</b>       | GMEM (Gibco™, 11710035) was stored at 4 °C upon receipt. To culture cells, a 5 mL aliquot of Gibco™ Penicillin-Streptomycin (10,000 U/mL) and 25 mL aliquot of FBS (Sigma) was added to a 500 mL bottle of GMEM which was subsequently stored at 4 °C.   |
| <b>Dulbecco's Modified Eagle Medium (DMEM)</b>      | DMEM (Gibco™ 41966052) was stored at 4 °C upon receipt. To culture cells, a 5 mL aliquot of Gibco™ Penicillin-Streptomycin (10,000 U/mL) and 25 mL aliquot of FBS (Sigma) was added to a 500 mL bottle of DMEM which was subsequently stored at 4 °C.    |
| <b>Eagle's Minimum Essential Medium (EMEM)</b>      | EMEM (Fisher, 50188268FP) was stored at 4 °C upon receipt. To culture cells, a 5 mL aliquot of Gibco™ Penicillin-Streptomycin (10,000 U/mL) and 25 mL aliquot of FBS (Sigma) was added to a 500 mL bottle of EMEM which was subsequently stored at 4 °C. |
| <b>Gibco™ Penicillin-Streptomycin (10,000 U/mL)</b> | Upon delivery, Gibco™ Penicillin-Streptomycin was aliquoted into 5 mL aliquots before being frozen at -20 °C.                                                                                                                                            |
| <b>Opti-MEM™ Reduced Serum Medium</b>               | Upon receipt, Opti-MEM™ (Gibco™ Opti-MEM™) was stored at 4 °C.                                                                                                                                                                                           |
| <b>FuGENE® 6 Transfection Reagent</b>               | Upon receipt, FuGENE® 6 Transfection Reagent (Promega, E2692) was stored at 4 °C.                                                                                                                                                                        |

|                                       |                                                                                                                                                                                                                                                                                                                                                                                                                                                  |
|---------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>Fetal Bovine Serum (FBS)</b>       | Fetal Bovine Serum (Sigma, F9665-500ML) was aliquoted into 25 mL aliquots upon delivery and subsequently stored at -20 °C.                                                                                                                                                                                                                                                                                                                       |
| <b>EndoGRO™-MV Complete Media Kit</b> | EndoGRO™-MV Complete Media Kit (Merck, SCME004) was purchased from Merck. The EndoGRO™-MV Complete Media Kit contained EndoGRO™ Basal Medium and the EndoGRO™-MV Supplement Kit which contained EndoGRO-LS Supplement, rh EGF, L-Glutamine, Hydrocortisone Hemisuccinate, Heparin Sulphate, Ascorbic Acid, and FBS. Upon receipt, rat tail and EndoGRO™ Basal Medium was stored at 4°C while the EndoGRO™-MV Supplement Kit was stored at -20°C. |
| <b>Collagen Type I Rat Tail</b>       | Collagen Type I rat tail (Merck, 08-115) was purchased from Merck and stored at 4°C upon receipt.                                                                                                                                                                                                                                                                                                                                                |

Table S1: Buffers and solutions used in this thesis.

## Appendix 2 - OLIGONUCLEOTIDE PRIMERS

| Primer       | Sequence 3'-5'                                                                                                    |
|--------------|-------------------------------------------------------------------------------------------------------------------|
| 61           | 5' TGAAAAACACGATGATAATACCAATGGTCTTCACACTCGAAG 3'                                                                  |
| 62           | 5' CATTTTGTATTCAACTTCTAACTTGTATTGCAGCTTATAATG 3'                                                                  |
| 60           | 5' TGGTATTATCATCGTGTTCCTTCAAAG 3'                                                                                 |
| 63           | 5' AGAAGTTGAATAACAAAATGCCG 3'                                                                                     |
| 55           | 5' GTACTGTTGGTAAAGCCACCCATGGAACCTGGACTTTC 3'                                                                      |
| 56           | 5' TCTTCGAGTGTGAAGACCATTCCATCCACAACCTTATATAG 3'                                                                   |
| 59           | 5' GTACTGTTGGTAAAGCCACCATGCAGGTTTCAGCTTAAAGAATC 3'                                                                |
| 57           | 5' ATGGTCTTCACACTCGAAG 3'                                                                                         |
| 58           | 5' GGTGGCTTTACCAACAGTAC 3'                                                                                        |
| 3            | 5' CATTGTAGGGGTGTTACATTTTTGCTGTTGACTTTGGGACATCTCGGATTTTATTGTC<br>TAGAGGGACTGAGGGGAGAGGTTTCGGTCAAAGGAACAAGTGATG 3' |
| 34           | 5' AGAAAAACAGACATTGTCAATTGCAAAATGAAAAAACTAACACCCCTCCTTCGAA<br>CCATCCCAAACATGCATGGAACCTGGACTTTC 3'                 |
| 1            | 5' CATGTTTGGGATGGTTCGAAAGGAGGGGTGTTAGTTTTTTTCATTGCAATTGACAA<br>TGTCTG 3'                                          |
| 4            | 5' CCGAACCTCTCCCCTCAGTCCCTCTAGACAATAAAATCCGAGATGTCCCAAAGTCAAC<br>AGCAAAAATGTAACACCCCTAC 3'                        |
| 30           | 5' GGAGACAACCAGGTTTTATGT 3'                                                                                       |
| 31           | 5' GTCGGACATAAAACCTGGTTGT 3'                                                                                      |
| 120          | 5' TATAAGGTTGTGGATGGAGCCACCATGGTGAGCAAG 3'                                                                        |
| 70           | 5'GAGGGACTGAGGGGAGAGGTTTCGGTTTACTTGTACAGCTCGTCCATG 3'                                                             |
| 119          | 5' TCCATCCACAACCTTATATAG 3'                                                                                       |
| 71           | 5' ACCGAACCTCTCCCCTCAG 3'                                                                                         |
| 64           | 5' TGCAAACCTCAGGTTATGTGGAG 3'                                                                                     |
| 65           | 5' TGCTCCTTTTAAAGACTTATATAG 3'                                                                                    |
| 79           | 5' CATCCCTCAAAGACCCCGGAAAGATGGTTCCTCAGGCTCTC 3'                                                                   |
| 80           | 5' TCGTTGAAAGGCTAGCCAGTCCTTACAGTCTGGTCTCACCCCC 3'                                                                 |
| 78           | 5' CTTTCCCGGGGTCTTTTG 3'                                                                                          |
| 81           | 5' TAAGGACTGGCTAGCCTTTC 3'                                                                                        |
| 74           | 5' TGTTTCCTGTGTGAAATTGTTATC 3'                                                                                    |
| 75           | 5' GCTATGACCATGATTACGCCAAGC 3'                                                                                    |
| 133          | 5' AAAAATGTAACACCCCTACACCGGGAAAGCCGCCACCATGGAACCTGGACTTTCTTG<br>3'                                                |
| 134          | 5' TAGTTTTTTTCATGATGGATATACACAATCCGTAGATTTCGGGCATTTTGTATTCAAC<br>TTCCCTAGCTTACTTGTACAGCTCGTC 3'                   |
| 113          | 5' TGTAGGGGTGTTACATTTTTG 5'                                                                                       |
| 135          | 5' ATCCATCATGAAAAAACTAACACCCCTCCTTTCGAACCATCCCAAACATGGATGCC<br>GACAAGATTG 3'                                      |
| cSN IKOV Fwd | 5' CTCAAAAGACCCCGGAAAGATGGCTCAGTTGGTCACTTTG 3'                                                                    |

|              |                                                  |
|--------------|--------------------------------------------------|
| cSN IKOV Rev | 5' GTTGAAAGGCTAGCCAGTCCCTAGAATGCAGAACTCTTG 3'    |
| 1840         | 5' TTTCACACAGGAAACAGCTATGACCATGATTACGCC 3'       |
| 1841         | 5' TCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATC 3'      |
| 146          | 5' TGATGATGTGCGGCCGCTCTTCACAGAGCAATGACTCCAAAG 3' |
| 157          | 5' CCTACACCGGGAAAGCCGATGATCCGCGACGTGGA 3'        |
| 158          | 5' GACGAACCTGTAGCCACGTCGTAGCAAACCACCAA 3'        |
| 159          | 5' TGTGAGGGTCTGGGCCATAGAACTGATGAGAGGGAG 3'       |
| 141          | 5' GCACTGAACACCCTTAAGGATAGC 3'                   |
| 144          | 5' AGAGCGGCCGCACATCAT 3'                         |
| 211          | 5' CCCTTATACTCAGCAGCCAGGTTTCAGCTTAAAGAAT 3'      |
| 210          | 5' ACTAAGCTTGAGATTAAGGCTGCTGAGTATAAGGG 5'        |

Table S2: Sequences of oligonucleotide primers used in this project.

## Appendix 3 - CLONING SCHEMATICS

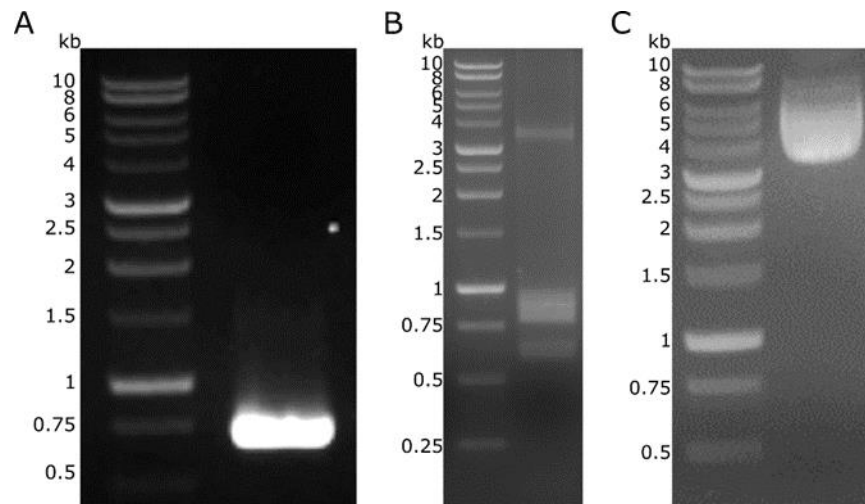


Figure A1: 1% Agarose gel of PCR products used to create pTIT-NLuc. A, shows NLuc (including the Poly(A) tail) amplified using oligonucleotide primers 61 and 62 (expected size 721 bp). B, shows the pTIT backbone as amplified using oligonucleotide primers 60 and 63. The correct sized band (3184 bp) was excised from the agarose gel and purified. After successful assembly and extraction from bacterial culture, C shows correctly assembled pTIT-NLuc (expected size 3862 bp). The sequences of all oligonucleotide primers shown are listed in Appendix 2.

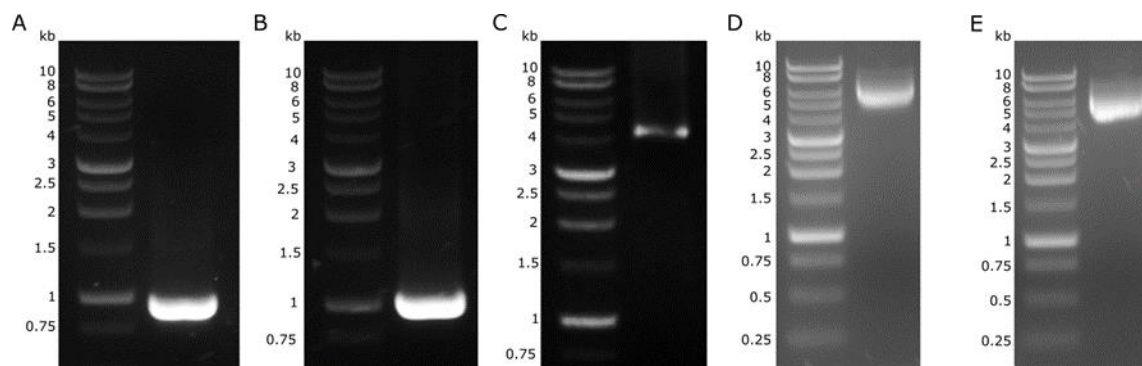


Figure A2: 1% Agarose gels of PCR products used to assemble pNL1.1 sec62-NLuc and pNL1.1 62-NLuc. Bands present in A and B show 62-71-3 amplified using either oligonucleotide primers 59 and 56 (expected size 860 bp) or 55 and 56 (expected size 913 bp), respectively. C, is the amplified PCR product of the pNL1.1 backbone using oligonucleotide primers 58 and 57 (expected size 3861 bp). Together, these form the two constructs pNL1.1 sec62-NLuc and pNL1.1 62-NLuc which, after successful assembly and extraction from bacterial culture, are shown in D and E (expected size 4789 bp and 4734 bp respectively). The sequences of all oligonucleotide primers shown are listed in Appendix 2.

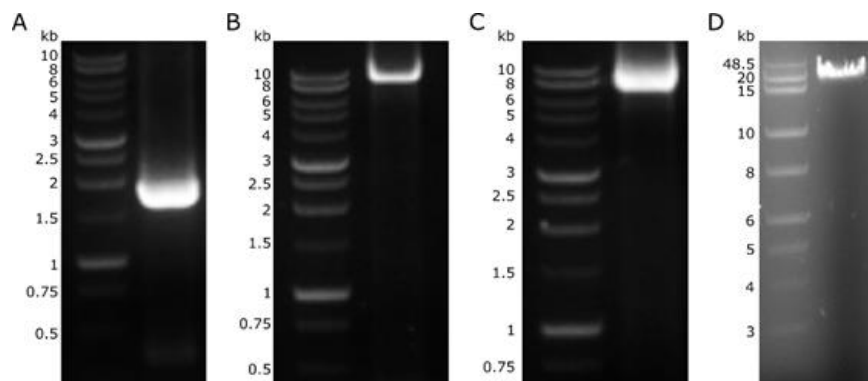


Figure A3: Agarose gels of the PCR products involved in the creation of RABV-62-H-E-DsR. A, (1% agarose gel), shows the PCR product of the 62-71-3 scFv cassette amplified by oligonucleotide primers 34 and 3 (expected size 1761 bp) while B and C (both 1% agarose) show the amplification of the RABV-cSN backbone using oligonucleotide primer pairs 1 and 30 (expected size 8264 bp) and 4 and 31 (expected size 7631 bp), respectively. The fully assembled construct, RABV-62-H-E-DsR has been run on a 0.3% agarose gel in D (expected size 17475). The sequences of all oligonucleotide primers shown are listed in Appendix 2.

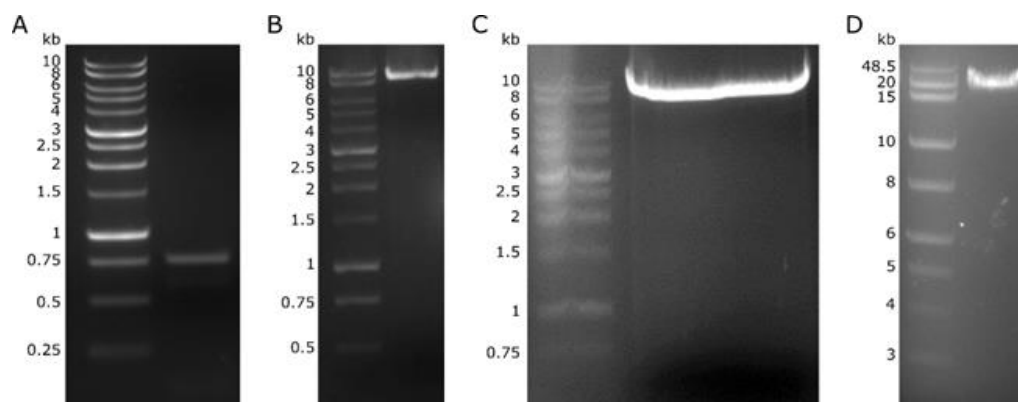


Figure A4: Agarose gels of the PCR products involved in the creation of RABV-62-H-E-mCH. A, shows a 1% agarose gel where mCHERRY has been amplified using oligonucleotide primers 120 and 70 (expected size 760 bp) while B and C show the amplification of the RABV-62-H-E-DsRED backbone on 1% agarose gels using oligonucleotide primer pairs 119 and 30 (expected size 9192 bp) and 71 and 31 (expected size 7632 bp). The assembled final product, RABV-62-H-E-mCH, is shown in a 0.3% agarose gel in D (expected size 17515 bp). The sequences of all oligonucleotide primers shown are listed in Appendix 2.

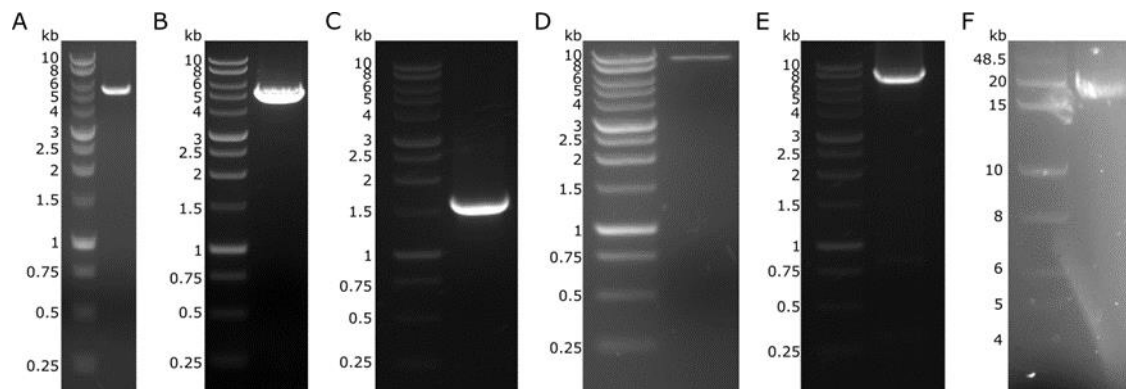


Figure A5: An agarose gel of the PCR products used in the point mutation of amino acid 226 from K to R. A, shows the PCR product of oligonucleotide primer 64 and 65 used to amplify and linearise pTIT-G (expected size 4932 bp). After successful assembly into the resulting construct named RABV-K226R-G (which is shown in B), the G gene was then amplified using oligonucleotide primers 79 and 80 and is shown in C (expected size 1622 bp). RABV-62-H-E-mCH was linearised in two parts using oligonucleotide primer pairs 78 and 74, and 81 and 75 and are shown in D and E (expected sizes 8579 bp and 7364 bp). PCR products from lanes B, C, and D were assembled to form RABV-62-H-E-mK which is shown in F (expected size 17515 bp). A 1% agarose gel was used in A-E, while 0.3% was used in F. The sequences of all oligonucleotide primers shown are listed in Appendix 2.

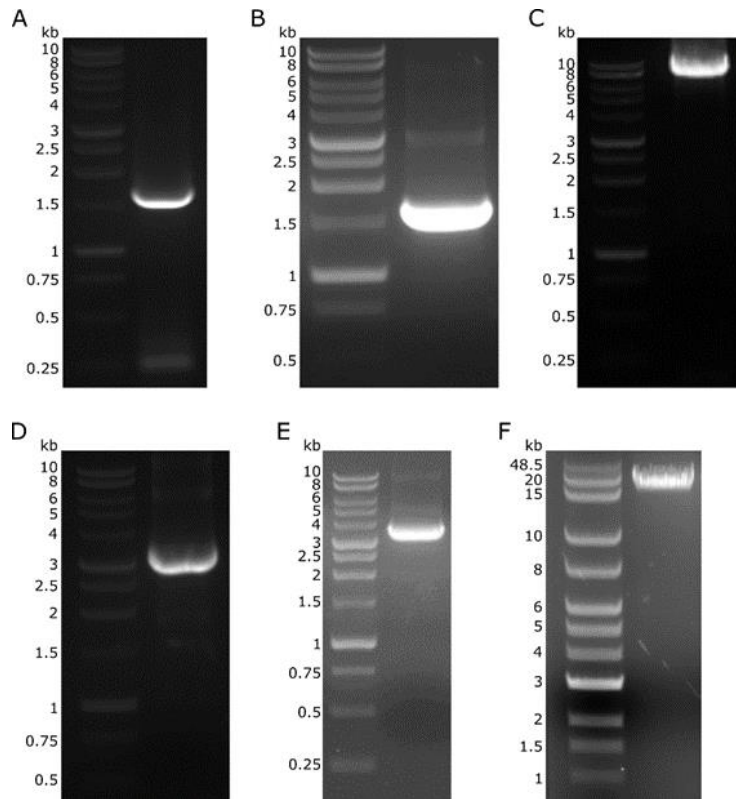


Figure A6: Agarose gels showing the PCR products involved in the re-insertion of the 62-71-3-mCHERRY cassette into RABV-cSN. A, shows the amplified PCR product of the 62-71-3-mCHERRY cassette using oligonucleotide primers 133 and 134 (expected size 1749 bp). B, shows the amplification of RABV-K226R-G using oligonucleotide primers 79 and 80 (expected size 1622 bp). C, D, and E show the linearisation of the RABV-cSN backbone using primer pairs 113 and 30, 81 and 31, and 78 and 135 (expected size, 8243 bp, 2732 bp, and 3294 bp respectively). As multiple bands are present in E, the correct sized band was excised and purified through gel extraction. Lane F shows the final assembled product, named RABV-62-H-E-mK-Poly(A) (expected size 17524 bp). A 1% agarose gel was used in A-E, while 0.3% was used in F. The sequences of all oligonucleotide primers shown are listed in Appendix 2.

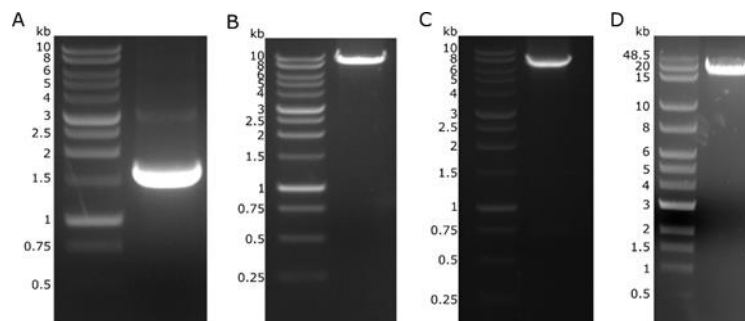


Figure A7: Agarose gel showing the PCR products used in the creation of RABV-62-H-E-mCH-IKOV. A, (1% agarose gel), shows amplification of IKOV G gene using oligonucleotide primers cSN IKOV Fwd and cSN IKOV Rev (expected size 1615 bp). B and C (1% agarose) show the PCR products of the linearisation of the RABV-62-H-E-Poly(A) backbone using oligonucleotide primer pairs 78 and 1841, and 81 and 1840 (expected size 8582 bp and 7380 bp respectively). D (0.3% agarose) shows the fully assembled product, RABV-62-H-E-mCH-IKOV (expected size 17505 bp). The sequences of all oligonucleotide primers shown are listed in Appendix 2.

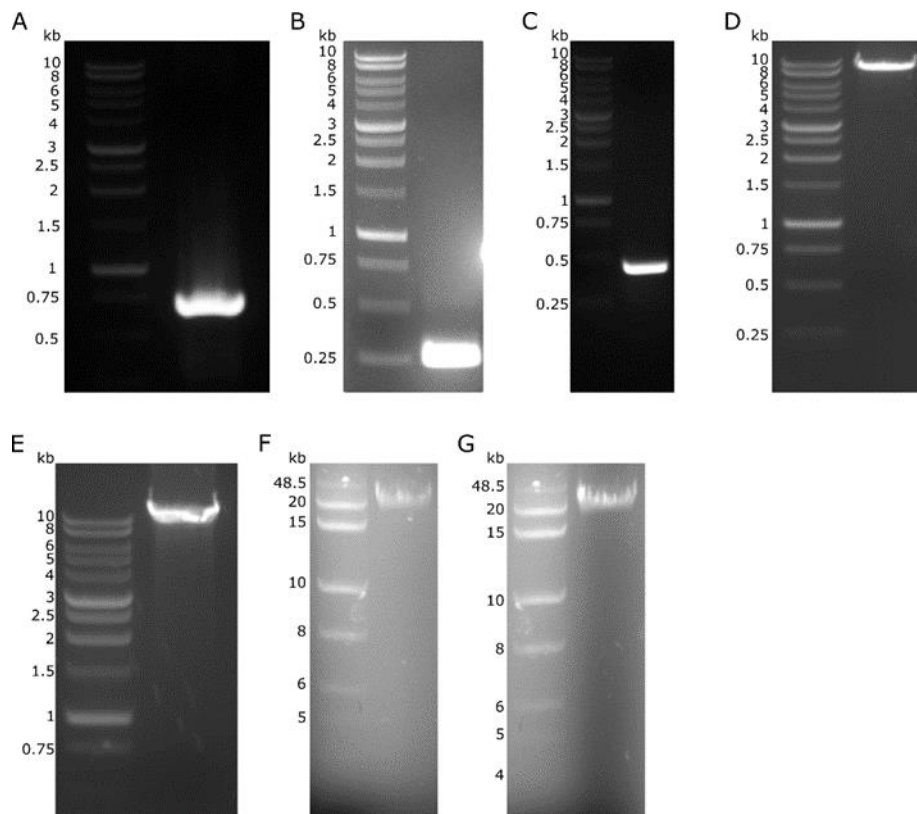


Figure A8: A schematic of the cloning used to create two TNF $\alpha$ -expressing RABV constructs, RABV-TNF $\alpha$ -H-E-mK and RABV-TNF $\alpha$ (mem)-H-E-mK. To create RABV-TNF $\alpha$ -H-E-mK, full-length TNF $\alpha$  was amplified using oligonucleotide primer pair 157 and 146 (Ai) and RABV-62-H-E-mK-Poly(A) linearised using oligonucleotide primer pairs 141/30, and 144/31 (B). These fragments were then assembled to create RABV-TNF $\alpha$ -H-E-mK (C). To create membrane-bound TNF $\alpha$  (TNF $\alpha$ (mem)) needed for RABV-TNF $\alpha$ (mem)-H-E-mK, bases critical for the post-translational cleavage of TNF $\alpha$  to allow secretion were removed by site-directed mutagenesis using oligonucleotide primer pair 159 and 158 (Aii). TNF $\alpha$ (mem) was then amplified using oligonucleotide primer pair 157 and 146 (Aii) and substituted into linearised RABV-62-H-E-mK-Poly(A) (B) to form RABV-TNF $\alpha$ (mem)-H-E-mK (C). All oligonucleotide primers have been colour coded to indicate homologous regions where purple indicates homology to TNF $\alpha$ , dark blue to the transcriptional stop unit, orange to the 8xHis tag, and grey to the RABV-62-H-E-mK-Poly(A) backbone. The sequences of all oligonucleotide primers shown are listed in Appendix 2.

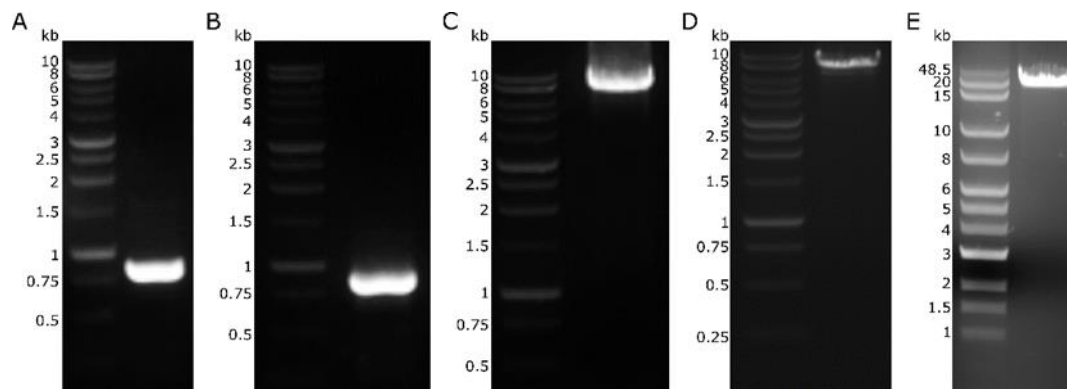


Figure A9: Agarose gel showing the PCR products involved in the creation of RABV-62scFv. A (1% agarose) shows amplification of the 62-71-3 scFv sequence using oligonucleotide primers 133 and 211 (expected size 834 bp) while B (1% agarose) shows amplification of mCHERRY using oligonucleotide primers 210 and 134 (expected size 863 bp). C and D (1% agarose gel) show the amplified PCR products generated when linearising the RABV-62-H-E-mK-Poly(A) backbone using oligonucleotide primer pair 113 and 30, and 135 and 31 (expected size 8243 bp and 7598 bp). As two bands are present in D, the correct sized band was excised and purified through gel extraction. Lastly, E (0.3% agarose) shows the fully assembled product RABV-62scFv (expected size 17437 bp). The sequences of all oligonucleotide primers shown are listed in Appendix 2.