Search for an antibody-based vehicle for delivering therapeutic molecules into the central nervous system

ROUX, Adrien

Abstract
The aim of my thesis was to try to identify novel pathways for trafficking molecules into the brain through the Blood Cerebrospinal Fluid Barrier and the central technology for identifying novel receptor-mediated transport pathways has been to use “in vivo phage display”. The first aim of the thesis was to develop the in vivo model to study the transport of phage across the BCSFB. The interesting candidate phage are those which bind to a transport receptor on the choroid plexus epithelium, and which are then passaged into the CSF. The other aim of the thesis was to implement an in vitro model of the BCSFB using primary epithelial cells obtained from the dissected rat choroid plexus. The in vitro model mimicking the BCSFB was implemented and validated with success. The candidates resulting from the in vivo panning experiments were then tested although unfortunately we were not able to show convincing transport of our reformatted IgGs across the choroid plexus epithelium in vitro. The reasons for this are discussed.

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Search for an antibody-based vehicle for delivering therapeutic molecules into the central nervous system

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La Faculté des sciences, sur le préavis de Monsieur J.-C. MARTINO, professeur ordinaire et directeur de thèse (Département de biologie cellulaire), Monsieur K. MAUNDRELL, docteur et codirecteur de thèse (Merck Serono S.A., Aubonne), Monsieur C. WEIDENFELDER, docteur (Merck Serono S.A., Aubonne), et Madame F. MILLER, docteure (Unité de pathogénie des infections systémiques, Institut National de la Santé et de la Recherche Médicale, Paris, France), autorise l'impression de la présente thèse, sans exprimer d'opinion sur les propositions qui y sont énoncées.

Genève, le 10 septembre 2014

Thèse - 4756 -

Le Doyen

N.B. - La thèse doit porter la déclaration précédente et remplir les conditions énumérées dans les "Informations relatives aux thèses de doctorat à l'Université de Genève".
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TABLE OF CONTENT

ACKNOWLEDGEMENT...........................................................................................................3
TABLE OF CONTENT .............................................................................................................5
LIST OF FIGURES ................................................................................................................11
LIST OF TABLES ..................................................................................................................15
LIST OF APPENDIX ............................................................................................................17
ABBREVIATIONS ................................................................................................................19
ABSTRACT ..........................................................................................................................23
RESUME ..............................................................................................................................27

INTRODUCTION ..................................................................................................................31

1. HISTORICAL REVIEW OF THE CONCEPT OF COMPARTMENTALISATION BETWEEN THE BLOOD AND THE CNS ....46
2. THE DIVERSITY OF BLOOD-CNS BARRIERS ........................................................................46
   2.1. THE BBB ..................................................................................................................46
   2.2. THE BCSFB .............................................................................................................46
   2.3. THE ARACHNOID BARRIER ....................................................................................46
   2.4. OTHER BARRIERS ..................................................................................................46
3. DESCRIPTION OF THE CNS FLUIDS .............................................................................46
   3.1. INTERSTITIAL CEREBRAL FLUID (ISF) .................................................................47
   3.2. CEREBROSPINAL FLUID (CSF) .............................................................................47
4. BCSFB DYSFUNCTION IN PATHOLOGIES AND AGEING .................................................49
5. PROBLEM OF DRUG DELIVERY: DIFFERENT STRATEGIES FOR ACCESSING THE BRAIN ..............................................50
   5.1. INVASIVE STRATEGIES ..........................................................................................51
   5.2. NON-INVASIVE STRATEGY AS ALTERNATIVE ROUTE ........................................52
6. TRANSPORT PATHWAYS INTO THE BRAIN ...................................................................54
   6.1. PASSIVE DIFFUSION ...............................................................................................55
   6.2. PARACELLULAR PATHWAY .....................................................................................55
   6.3. CARRIER MEDIATED TRANSPORTER (CMT) .........................................................55
   6.4. RECEPTOR MEDIATED TRANCYTOSIS (RMT) .....................................................56
   6.5. CHOICE OF THE POSITIVE CONTROL FOR RMT ................................................57
7. PHAGE DISPLAY TECHNOLOGIES ...........................................................................57
   7.1. STRUCTURE OF A FILAMENTOUS PHAGE .................................................57
   7.2. BIOLOGY OF THE FILAMENTOUS PHAGE ..............................................58
   7.3. THE BIOPANNING CONCEPT AND GENERAL PRINCIPLES OF THE PHAGE DISPLAY APPROACH .................................................................59
   7.4. APPLICATION OF PHAGE DISPLAY TO NEUROBIOLOGY .......................62
   7.5. IN VIVO PHAGE DISPLAY ..........................................................................62

8. OUR APPROACH ....................................................................................................63
   8.1. IN VIVO PHAGE DISPLAY TO INVESTIGATE TRANSPORT ACROSS THE BCSFB ............................................................63
   8.2. RATIONALE FOR STUDYING THE BCSFB .....................................................64
   8.3. IN VITRO MODEL TRANSPORT OF THE BCSFB ...........................................65
   8.4. CHARACTERIZATION OF THE IN VITRO MODEL ........................................66

MATERIAL AND METHODS ......................................................................................67

1. MOLECULAR BIOLOGY .........................................................................................67
   1.1. MATERIAL FOR MOLECULAR BIOLOGY ......................................................67

1.2. METHOD OF CDNA PRODUCTION BY RT-PCR ..............................................68
   1.2.1. RNA isolation.........................................................................................68
   1.2.2. Reverse Transcription ..........................................................................68
   1.2.3. Polymerase Chain Reaction ................................................................69
   1.2.4. Agarose gel electrophoresis ..................................................................70
   1.2.5. DNA Clean-up .......................................................................................71
   1.2.6. Determination of nucleotide concentration ..............................................72

1.3. METHOD OF CDNA CLONING INTO PLASMID VECTORS ..............................73
   1.3.1. Enzymatic digestion ..............................................................................73
   1.3.2. Dephosphorylation of the vector ................................................................73
   1.3.3. Ligation ................................................................................................74
   1.3.4. Transformation by electroporation .........................................................74
   1.3.5. DNA plasmid preparation .....................................................................75
   1.3.6. DNA sequencing using the DNA sequencer robotic system ....................76
   1.3.7. Sequences analysis ................................................................................76

1.4. DETAIL OF THE DIFFERENT CLONINGS OF OX-26 .................................77
   1.4.1. Cloning of VL OX-26 and VH OX-26 from OX-26 hybridoma cells into pBluescript vector ........................................77
   1.4.2. Cloning VL OX-26 and VH OX-26 of into pTT5 vectors to express recombinant OX-26 ................................................81
   1.4.3. Cloning of scFv OX-26 into pHal14 .........................................................83
2. PRODUCTION AND PURIFICATION OF RECOMBINANT PROTEINS ................................................................. 85
   2.1. MATERIAL FOR PROTEIN PRODUCTION AND PURIFICATION .............................................................. 85
   2.2. CULTURE OF HEK293 ................................................................................................................................ 86
   2.3. TRANSIENT TRANSFECTION OF IgG WITH THE VIRUS ............................................................................. 86
   2.4. CULTURE OF OX-26 HYBRIDOMA.............................................................................................................. 86
   2.5. PROTEIN PURIFICATION ............................................................................................................................. 87
       2.4.1. Purification of 6-HIS tagged proteins ..................................................................................................... 87
       2.4.2. Purification of IgG with protein A ......................................................................................................... 87
   2.6. PRODUCTION AND PURIFICATION OF HYBRIDOMA OX-26 ................................................................. 88
   2.7. PRODUCTION AND PURIFICATION OF RECOMBINANT OX-26 ............................................................ 89

3. PHAGE DISPLAY ............................................................................................................................................... 89
   3.1. MATERIAL AND PREPARATION OF THE REAGENTS FOR PHAGE DISPLAY ........................................ 89
   3.2. METHODS .................................................................................................................................................. 91
       3.2.1. Construction of phagemids for positive phage controls ....................................................................... 91
       3.2.2. Bacteria culture and long term storage ................................................................................................. 92
       3.2.3. Rescue of the phage ............................................................................................................................. 93
       3.2.4. Phage concentration ............................................................................................................................ 94
       3.2.5. Lipopolysaccharide elimination .......................................................................................................... 94
       3.2.6. Phage Titration ..................................................................................................................................... 95
       3.2.7. Recovery of internalized phage from a cell ........................................................................................ 95
       3.2.8. Bacterial infection with phage ............................................................................................................ 96
       3.2.9. Reformating scFv into pTT vector ...................................................................................................... 96
   3.3. PRODUCTION OF scFvs OX-26 PHAGE .................................................................................................. 99

4. IN VIVO MODEL .............................................................................................................................................. 99
   4.1. MATERIAL FOR IN VIVO MODEL .................................................................................................... 99
   4.2. IN VIVO METHODS ............................................................................................................................... 99
       4.2.1. Animal welfare and origin of the animals ......................................................................................... 99
       4.2.2. Anaesthesia ....................................................................................................................................... 100
       4.2.3. Product administration into the peripheral circulation .................................................................. 100
       4.2.4. Blood sampling .................................................................................................................................. 101
       4.2.5. CSF Sampling technique .................................................................................................................. 101
       4.2.6. Dissection .......................................................................................................................................... 102

5. IN VITRO TRANSPORT MODELS .................................................................................................................. 103
   5.1. MATERIAL ................................................................................................................................................ 103
5.2. **IN VITRO METHODS** ........................................................................................................ 104
  5.2.1. Primary culture from choroid plexus epithelial cells ................................................. 104
  5.2.2. Culture of choroidal epithelial Z310 cells .................................................................. 107
  5.2.3. Dual chamber system and transport assay ................................................................. 107
  5.2.4. Measure of the TEER ............................................................................................... 108

6. **BIOCHEMICAL METHODS** .............................................................................................. 109
  6.1. Material .......................................................................................................................... 109
  6.2. Extraction of membrane proteins .................................................................................. 109
  6.3. Gel electrophoresis and immunobloting ....................................................................... 111
  6.4. N-terminal sequencing .................................................................................................. 111

7. **IMMUNOLOGICAL METHODS** ......................................................................................... 112
  7.1. Material ........................................................................................................................ 112
  7.2. FACS ................................................................................................................................ 112
  7.3. Detection and quantification of human IgG by ELISA .................................................. 113
  7.4. Immunohistochemistry (IHC) and immunocytochemistry (ICC) ................................. 114
    7.4.1. Tissue preparation and sectioning ............................................................................ 114
    7.4.2. Detection with fluorescence labelled antibodies .................................................... 115
    7.4.3. Detection with immunoperoxidase staining (DAB-ABC-Method) ....................... 115

RESULTS ....................................................................................................................................... 117

1. **CHOICE OF THE CONTROLS** ........................................................................................... 117
  1.1. Identification of known RMTs on CP ............................................................................ 117
  1.2. Anti-rat transferrin receptor antibody, OX-26 ............................................................ 119
    1.2.1. N-Sequencing of OX-26 ....................................................................................... 120
    1.2.2. Binding of hybridoma OX-26 and recombinant OX-26 to Z310 cells as tested by FACS ........................................................................................................... 121
    1.2.3. Testing the binding of recombinant OX-26 by ICC ............................................. 123
    1.2.4. Test of binding of the scFv OX-26 phage to Z310 cells ........................................ 123
  1.3. Construction of additional phage controls .................................................................... 125
    1.3.1. Cloning of the Angiopep peptide ....................................................................... 126
    1.3.2. Cloning of rat leptin ......................................................................................... 127
    1.3.3. Cloning of one antibody fragment control: FCS .............................................. 128
  1.4. Low density lipoprotein-related protein 2 (LRP2) ....................................................... 129

2. **IN VIVO MODEL** .............................................................................................................. 130
  2.1. Set up of the in vivo phage display ............................................................................... 130
    2.1.1. Phage injection ...................................................................................................... 130
    2.1.2. Preparation of the phage for injection: elimination of LPS .................................. 131
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1.3. Effect of heparinated blood, serum and plasma, on phage infectivity</td>
<td>132</td>
</tr>
<tr>
<td>2.1.4. Time course for in vivo panning</td>
<td>132</td>
</tr>
<tr>
<td>2.2. In vivo phage display and biopanning results</td>
<td>133</td>
</tr>
<tr>
<td>2.2.1. The initial strategy</td>
<td>133</td>
</tr>
<tr>
<td>2.2.2. Quantification of the level of blood contamination in CSF</td>
<td>134</td>
</tr>
<tr>
<td>2.2.3. Approaches to optimise CSF sampling</td>
<td>134</td>
</tr>
<tr>
<td>2.2.4. Recovery of phage from CSF using the long-term canulation model</td>
<td>137</td>
</tr>
<tr>
<td>1.4.3.1. First round of in vivo panning</td>
<td>137</td>
</tr>
<tr>
<td>1.4.3.2. Second and third round of in vivo panning on canulated rats</td>
<td>137</td>
</tr>
<tr>
<td>2.2.5. Strategy using canulated rat and polyvalent phage rescue using hyperphage</td>
<td>138</td>
</tr>
<tr>
<td>2.3. Conclusion on in vivo phage display panning</td>
<td>139</td>
</tr>
<tr>
<td>3. Reformating of potential candidates</td>
<td>140</td>
</tr>
<tr>
<td>3.1. Reformating scFv V\text{H} and V\text{L} regions into the PTT vectors for co-expression in HEK cells</td>
<td>140</td>
</tr>
<tr>
<td>3.2. Production and purification</td>
<td>142</td>
</tr>
<tr>
<td>3.3. Western blotting of the candidate antibodies against membrane proteins from Z310 cells</td>
<td>143</td>
</tr>
<tr>
<td>3.4. Characterization of the candidate antibodies by IHC on tissue sections from rat brain</td>
<td>145</td>
</tr>
<tr>
<td>4. In vitro models of the BCSFB</td>
<td>147</td>
</tr>
<tr>
<td>4.1. Setup of the choroid plexus primary cells</td>
<td>147</td>
</tr>
<tr>
<td>4.1.1. Determination of the optimal coating of the well</td>
<td>147</td>
</tr>
<tr>
<td>4.1.2. Determination of the optimal seeding density</td>
<td>148</td>
</tr>
<tr>
<td>4.1.3. Optimisation of the size of the cluster of primary cells</td>
<td>149</td>
</tr>
<tr>
<td>4.1.4. Determination of the optimal time of culture</td>
<td>151</td>
</tr>
<tr>
<td>4.2. Further characterization of primary cell cultures</td>
<td>151</td>
</tr>
<tr>
<td>4.2.1. Presence of tight junction markers</td>
<td>151</td>
</tr>
<tr>
<td>4.2.2. Measurement of the capacitance and resistivity</td>
<td>152</td>
</tr>
<tr>
<td>4.2.3. Presence of known transporters on primary cells control by ICC and IHC</td>
<td>154</td>
</tr>
<tr>
<td>4.3. In vitro model using Z310 CPE cell line</td>
<td>155</td>
</tr>
<tr>
<td>4.3.1. Determination of the optimal seeding density of Z310.</td>
<td>155</td>
</tr>
<tr>
<td>4.3.2. Measure of the resistivity of Z310</td>
<td>156</td>
</tr>
<tr>
<td>5. In vitro transport experiments with reformatted candidate antibodies</td>
<td>157</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>163</td>
</tr>
<tr>
<td>REFERENCE LIST</td>
<td>171</td>
</tr>
<tr>
<td>APPENDIX</td>
<td>179</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

FIGURE 1: DIFFERENT CLASSES OF TRANSPORTERS PRESENT AT THE BLOOD-CNS BARRIERS .......................................................... 46
FIGURE 2: REPRESENTATION OF THE EXPERIMENT PERFORMED BY GOLDMANN ................................................................. 46
FIGURE 3: AUTORADIOGRAPHY OF ADULT MOUSE AFTER IV INJECTION OF RADIOLABELLED HISTAMINE ................................. 46
FIGURE 4: MAIN BARRIERS AND INTERFACE RESTRICTING FLUID MOVEMENT INTO AND WITHIN THE BRAIN ........................................ 46
FIGURE 5: NEUROVASCULAR UNIT STRUCTURE .......................................................................................................................... 46
FIGURE 6: STRUCTURE OF TIGHT JUNCTION AT THE BBB LEVEL .................................................................................................... 46
FIGURE 7: LOCALISATION OF THE CPs WITHIN THE BRAIN ........................................................................................................... 46
FIGURE 8: LOCALISATION AND STRUCTURE OF THE CP FROM THE LV ......................................................................................... 46
FIGURE 9: MENINGES VIEW .............................................................................................................................................................. 46
FIGURE 10: LOCALISATION OF THE CIRCUMVENTRICULAR ORGANS ........................................................................................... 46
FIGURE 11: CSF FLOW THROUGH THE HUMAN CNS ..................................................................................................................... 48
FIGURE 12: REABSORPTION OF THE CSF VIA THE CRANIAL SUB ARACHNOID SPACE (SAS) .......................................................... 49
FIGURE 13: OVERVIEW OF APPROACHES TO CNS DRUG DELIVERY ............................................................................................. 51
FIGURE 14: REPRESENTATION OF A BRAIN DELIVERY VECTOR .................................................................................................... 53
FIGURE 15: TRANSPORT PATHWAYS AT THE CNS BARRIER ........................................................................................................... 55
FIGURE 16: STRUCTURE OF A M13PHAGE ....................................................................................................................................... 58
FIGURE 17: LIFE CYCLE OF FILAMENTOUS M13 PHAGE ................................................................................................................ 59
FIGURE 18: PHAGE DISPLAY BIOPANNING ...................................................................................................................................... 60
FIGURE 19: PHAGE VECTOR; MAP OF THE PHAGEMID .................................................................................................................. 61
FIGURE 20: ANTIBODY STRUCTURE AND FRAGMENTS COMMONLY USED IN PHAGE DISPLAY .................................................. 62
FIGURE 21: STRATEGY TO IDENTIFY PHAGE ENCODED ANTIBODIES ABLE TO ENTER THE CSF ...................................................... 64
FIGURE 22: NANODROP SPECTROPHOTOMETER EQUIPMENT ...................................................................................................... 72
FIGURE 23: GENE PULSER EQUIPMENT FROM BIO RAD ............................................................................................................... 75
FIGURE 24: QUALITY OF RNA CHECKED ON 1.2% AGAROSE GEL .................................................................................................. 78
FIGURE 25: 0.8% AGAROSE GEL OF RT-PCR PRODUCTS .................................................................................................................... 79
FIGURE 26: 0.8% AGAROSE GEL TO CONTROL THE QUALITY OF THE DIGESTED INSERTS ............................................................... 79
FIGURE 27: DNA SEQUENCE OF THE VARIABLE REGION HEAVY CHAIN OX-26 .......................................................... 80
FIGURE 28: AMINO ACID SEQUENCE OF VARIABLE REGION HEAVY CHAIN OX-26 ........................................................ 80
FIGURE 29: DNA SEQUENCE OF THE VARIABLE REGION KAPPA LIGHT CHAIN OX-26 ............................................................. 80
FIGURE 30: AMINO ACID SEQUENCE OF THE VARIABLE REGION KAPPA LIGHT CHAIN OX-26 ...................................................... 80
FIGURE 31: 0.8% AGAROSE GELS SHOWING THE VL AND VH PCR PRODUCTS FROM OX-26 ............................................................ 81
FIGURE 32: VECTOR pTT5 WITH THE VH AND VKL OF OX-26 INSERT .............................................................................................. 82
FIGURE 33: STRATEGY OF THE PCRS TO OBTAIN scFv OX-26 PHAGEMID .................................................................................... 84
FIGURE 34: 0.8% AGAROSE GELS TO CLONE scFVS OX-26 INTO pHAL14 VECTOR ........................................................................... 85
LIST OF TABLES

Table 1: Main receptor and ligands used for drug delivery .................................................................57
Table 2: Preparation of the solutions needed for the reverse transcriptase reaction ..........................69
Table 3: PCR Program used for AmpliTaq ..........................................................................................70
Table 4: PCR Program used for Pwo .................................................................................................70
Table 5: Recommended percentage of agarose versus DNA size .......................................................71
Table 6: PCR program for sequencing ...............................................................................................76
Table 7: Primers used to PCR out the OX-26 sequence ...................................................................78
Table 8: PCR preparation for pTTS cloning ........................................................................................81
Table 9: Ligation of the VkL and the Vδi insert into pTTS vectors ....................................................82
Table 10: Strategy of the PCRs ..........................................................................................................83
Table 11: PCR mix for the amplification of the variable lambda region and the heavy chain of OX-26 .................................................................97
Table 12: PCR program for the amplification of the variable lambda region and the heavy chain of OX-26 .................................................................97
Table 13: Digestion mix of the PCR product ......................................................................................98
Table 14: Calculation of the amount of insert for the ligation ............................................................98
Table 15: Binding of anti-LRP2 antibody detected by FACS .............................................................119
Table 16: Amino acid N-sequencing results ......................................................................................120
Table 17: Sequence of the degenerate primers for the OX-26 variable regions ...............................121
Table 18: List of the control phagemid produced ..............................................................................126
Table 19: Candidates from the round 1 output with canulated rats ................................................137
Table 20: Analyze of the new round 2 ..............................................................................................139
Table 21: Primers and pool of primers used for the reformatting ....................................................140
Table 22: PCR conditions for reformatting ......................................................................................141
Table 23: Concentration of the reformatted antibody (candidates A to Q) ........................................142
LIST OF APPENDIX

APPENDIX 1: TABLE OF THE NUCLEOTIDE ABBREVIATION ACCORDING TO THE IUPAC ................................................................. 179
APPENDIX 2: CLONING OF LRP2 FROM KIDNEY AND CHOROID PLEXUS ................................................................................ 179
APPENDIX 3: AMINO ACID SEQUENCE OF THE scFv WITH NON-MODIFIED LINKER .............................................................. 180
APPENDIX 4: DNA OF THE GS LINKER USED FOR THE scFv ......................................................................................................... 180
APPENDIX 5: DNA SEQUENCE OF rLEPTIN IN pHA14 VECTOR: ................................................................................................. 180
APPENDIX 6: GENOTYPE OF DIFFERENT BACTERIA USED ........................................................................................................ 182
APPENDIX 7: DNA SEQUENCE OF FC5 ........................................................................................................................................ 182
APPENDIX 8: AMINO ACID SEQUENCE OF FC5 .......................................................................................................................... 182
APPENDIX 9: DNA SEQUENCE FROM FC44 ................................................................................................................................ 183
APPENDIX 10: AMINO ACID SEQUENCE OF FC44 .................................................................................................................. 183
APPENDIX 11: SEQUENCED DNA FROM THE LIGHT CHAIN FORWARD OF OX-26 ............................................................ 183
APPENDIX 12: SEQUENCED DNA FROM THE LIGHT CHAIN REVERSE OF OX-26 .............................................................. 184
APPENDIX 13: SEQUENCED DNA FROM HEAVY CHAIN FORWARD OF OX-26 ................................................................. 184
APPENDIX 14: SEQUENCED DNA FROM THE HEAVY CHAIN REVERSE OF OX-26 ............................................................... 185
APPENDIX 15: RESTRICTION ENZYMES USED ......................................................................................................................... 185
APPENDIX 16: mRNA SEQUENCE OF MOUSE LRP2 .................................................................................................................. 186
APPENDIX 17: AMINO ACID SEQUENCE OF LRP2 ..................................................................................................................... 190
APPENDIX 18: NUCLEOTIDIC SEQUENCE OF THE PRIMERS .................................................................................................... 193
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2YT</td>
<td>Yeast Extract and Triptone medium</td>
</tr>
<tr>
<td>3V</td>
<td>Third ventricle</td>
</tr>
<tr>
<td>4V</td>
<td>Fourth ventricle</td>
</tr>
<tr>
<td>AAALAC</td>
<td>Association for assessment and accreditation of laboratory animal care international</td>
</tr>
<tr>
<td>ABC</td>
<td>Adenosine triphosphate binding cassette</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer disease</td>
</tr>
<tr>
<td>AF</td>
<td>Alexa fluor</td>
</tr>
<tr>
<td>apoE</td>
<td>Apolipoprotein E</td>
</tr>
<tr>
<td>AU</td>
<td>Absorbance unit</td>
</tr>
<tr>
<td>AVP</td>
<td>Arginine vasopressin</td>
</tr>
<tr>
<td>Aβ</td>
<td>Amyloid beta</td>
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<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
</tr>
<tr>
<td>BBIs</td>
<td>Blood brain interfaces</td>
</tr>
<tr>
<td>BCNSB</td>
<td>Brain central nervous system barriers</td>
</tr>
<tr>
<td>BCRP</td>
<td>Breast cancer resistance-associated protein</td>
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<tr>
<td>BCSFB</td>
<td>Blood-cerebrospinal fluid barrier</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>BDT</td>
<td>Big dye terminator</td>
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<tr>
<td>BEC</td>
<td>Brain endothelial cells</td>
</tr>
<tr>
<td>BM</td>
<td>Basement membrane</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BSCB</td>
<td>Blood–spinal cord barrier</td>
</tr>
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<td>BSK</td>
<td>pBluescript SK vector</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CDR</td>
<td>Complementarity determining regions</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CP</td>
<td>Choroid plexus</td>
</tr>
<tr>
<td>CPe</td>
<td>Choroid plexus epithelial</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>CVOs</td>
<td>Circumventricular organs</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3′-diaminobenzidine</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diéthyl pyrocarbonate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco modified Eagle’s minimal essential medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxyribonucleotide</td>
</tr>
<tr>
<td>DSIP</td>
<td>Delta sleep-inducing peptide</td>
</tr>
</tbody>
</table>
DTR  Diptheria toxin receptor
DTT  Dithiothreitol
EAE  Experimental autoimmune encephalomyelitis
ECACC European Collection of Cell Culture
ECM  Extracellular matrix
EDTA Ethylenediaminetetraacetic acid
EGF  Epidermal growth factor
ELISA Enzyme-Linked Immunosorbent Assay
EPO  Erythropoietin
ERT  Enzyme replacement therapy
ESAM Endothelial selective adhesion molecule
EWB  ELISA washing buffer
FBS  Fetal bovine serum
Fc  Fragment constant
FITC Fluorescence isothiocyanate
Fwd  Forward
GABA γ-Aminobutyric acid
GDNF Glial-derived neurotrophic factor
GFAP Glial fibrillary acidic protein
GFP  Green fluorescent protein
GLUT Glucose transporter
GS  Peptidic linker
HC  Heavy chain
HEK Human embryonic kidney
HIV  Human immunodeficiency virus
ICF Interstitial cerebral fluid
ICV  Intracerebroventricular
ID  Injected dose
IGF-1  Insulin-like growth factor 1
IgG  Immunoglobulin G
IPTG Isopropyl β-D-1-thiogalactopyranoside
IR  Insulin receptor
ISF  Interstitial Fluid
IV  Intravenous
JAM  Junctional adhesion molecules
LacZ Gene from the lactose operon
LAT  L-amino acid transporters
LB  Lysogeny broth
LC  Light chain
LDL Low density lipoprotein
LIF Leukaemia inhibitory factor
LPS Lipopolysaccharide
LRP  **Low density lipoprotein receptor-related protein**
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>LSD</td>
<td>Lysosomal storage disorder</td>
</tr>
<tr>
<td><strong>LV</strong></td>
<td><strong>Lateral ventricle</strong></td>
</tr>
<tr>
<td>MCT</td>
<td>Monocarboxylate transporter</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean fluorescence intensity</td>
</tr>
<tr>
<td>MRPs</td>
<td>Multidrug resistance-associated proteins</td>
</tr>
<tr>
<td>MTH</td>
<td>Molecular Trojan Horse</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>OATPs</td>
<td>Organic anion transporting polypeptide</td>
</tr>
<tr>
<td>OATs</td>
<td>Organic anion transporters</td>
</tr>
<tr>
<td>OCTs</td>
<td>Organic cations transporters</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>O/N</td>
<td>Overnight</td>
</tr>
<tr>
<td>OVC</td>
<td>Office Cantonal Vétérinaire</td>
</tr>
<tr>
<td>PBCA</td>
<td>Polybutylcyanoacrylate</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson's disease</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PET</td>
<td>Polyethylene terephthalate</td>
</tr>
<tr>
<td>P-gp</td>
<td>P-glycoprotein</td>
</tr>
<tr>
<td>RAGE</td>
<td>Receptor for advanced glycation end products</td>
</tr>
<tr>
<td>RAP</td>
<td>Receptor associated protein</td>
</tr>
<tr>
<td>RBC</td>
<td>Red Blood Cells</td>
</tr>
<tr>
<td>Rev</td>
<td>Reverse</td>
</tr>
<tr>
<td>RMT</td>
<td>Receptor mediated transport</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute medium</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>SAS</td>
<td>Subarachnoid space</td>
</tr>
<tr>
<td><strong>scFv</strong></td>
<td><strong>Single-chain variable fragment</strong></td>
</tr>
<tr>
<td><strong>sdAb</strong></td>
<td><strong>Single domain antibody</strong></td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEC</td>
<td>Size exclusion chromatography</td>
</tr>
<tr>
<td>SLC</td>
<td>Solute carriers</td>
</tr>
<tr>
<td>SOC</td>
<td>Super optimal broth with catabolite repression (with glucose)</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris base, acetic acid and EDTA buffer</td>
</tr>
<tr>
<td>TB</td>
<td>Terrific broth</td>
</tr>
<tr>
<td>TfR</td>
<td>Transferin receptor</td>
</tr>
<tr>
<td>TG1</td>
<td>Termite group 1 (E.coli bacteria)</td>
</tr>
<tr>
<td>TJ</td>
<td>Tight junction</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>Tm</td>
<td>Melting temperature</td>
</tr>
<tr>
<td>TTR</td>
<td>Transthyretin</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra violet</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>(V_H)</td>
<td>Variable region of the heavy chain of immunoglobulin</td>
</tr>
<tr>
<td>(V_L)</td>
<td>Variable region of the light chain of immunoglobulin, could be kappa ((V_K)) or lambda ((V_\lambda))</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume/volume</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
</tr>
<tr>
<td>ZO</td>
<td>Zona occluden</td>
</tr>
</tbody>
</table>
ABSTRACT

The brain is a highly protected organ enclosed by several distinct barriers which maintain the delicately balanced environment necessary for correct neuronal function. The two most notable barriers are the Blood Brain Barrier (BBB) and the Blood Cerebrospinal Fluid Barrier (BCSFB) formed by epithelial cells of the choroid plexus. The great majority of molecules cannot cross these barriers and only very few small liposoluble molecules are able to enter the brain passively, while essentially all macromolecules are excluded. Thus the needs of the brain to receive nutrients and other factors from the blood, and to eliminate metabolic wastes resulting from brain activity are satisfied by highly specific transport systems. One mechanism of transport known as receptor mediated transport (RMT) involves vesicle-mediated transcytosis through the cell and is dedicated to the transport of larger molecules such as peptides and proteins. Several such receptors have been identified and have been targetted as potential routes of accessing the brain with therapeutic molecules for CNS diseases.

The aim of my thesis was to try to identify novel pathways for trafficking molecules into the brain, with the longer term aim of developing a vehicle able to deliver drugs to treat CNS diseases. My project has focussed on the BCSFB, and the central technology for identifying novel receptor-mediated transport pathways has been to use “in vivo phage display”. For this approach, a large library of bacteriophage each displaying a specific antibody is used. The library is injected into the peripheral bloodstream and the CSF is then sampled. Any phage present in the CSF are recovered and the encoded antibody is identified. By looking for enrichment of specific sequences in this population we hope to identify antibodies which have been able to cross the BCSFB by interacting with a functional transport receptor. This approach makes no prior assumptions about the nature of the receptor being targetted and thus potentially, allows the discovery of novel transport systems into the brain.

In order to validate the in vivo screening results and investigate further the transport of the phage encoded antibody candidates, an in vitro model of the BCSFB was established using either primary rat choroid plexus epithelial cells or, more conveniently, an established cell line, Z310 (a kind gift from Dr. Zheng, Perdue University).
The aim of the first part of the thesis was to develop the *in vivo* model to study the transport of phage across the BCSFB. For this, a number of negative and positive control phage were constructed and produced. As negative controls we have prepared 'empty' phage expressing the unmodified gIII protein, as well as recombinant phage which express the irrelevant antibodies, anti-hMCP1 and anti-hTIE2 neither of which can cross-react with the corresponding rodent proteins. As positive controls we have constructed phage expressing the anti-rat transferrin receptor, OX-26, the rat EGF (ligand), or a single domain antibody (sdAb), FC5 which has been shown to cross the BBB. These sequences were fused to the phage gIII gene and are thus expressed on the surface of the phage. Recombinant phage are injected into the tail vein of the rat and allowed to circulate though the body for different times before harvesting and analysis of the CSF.

Biopanning for binders to the choroid plexus was performed following the same protocol but using a large phage antibody library in which the heavy and light antibody chains are expressed as a single polypeptide separated by a flexible linker element. In this case, non specific binders are eliminated during passage through the peripheral vascular system while the remaining phage, substantially depleted for potential 'false positives' have a higher chance of identifying receptors specific for the choroid plexus. The interesting candidate phage are those which bind to a transport receptor on the choroid plexus epithelium, and which are then passaged into the CSF. Such phage are likely to be rare, however the *in vivo* system provides a powerful selection for positive phage candidates.

A major technical issue has been the difficulty in avoiding trace amounts of contaminating blood during collection of the CSF, since this represents a major source of false positive phage. Different protocols were tested and finally we were able to overcome this problem using a model of long-term canulation of the cisterna magna. Several rounds of *in vivo* biopanning identified candidates which where then reformatted as normal IgG antibodies and characterized further.

The aim of the second part of the thesis was to implement an *in vitro* model of the BCSFB using primary epithelial cells obtained from the dissected rat choroid plexus. The procedure was adapted from a published protocol. In this model, cells are seeded on transwell membranes in a dual chamber culture system and after 7 days in culture show many of the biophysical and biochemical properties of the *in vivo* epithelium. In parallel, the rat choroid plexus epithelial cell line Z310 was used as an alternative to the primary cells. The
candidates were tested using these two *in vitro* models as well as in a classical cell binding approach.

The *in vitro* model mimicking the BCSFB was implemented and validated with success. The candidates resulting from the *in vivo* panning experiments were then tested although unfortunately we were not able to show convincing transport of our reformatted IgGs across the choroid plexus epithelium *in vitro*. The reasons for this are discussed.
RESUME

Le cerveau est un organe très protégé entouré par plusieurs barrières distinctes qui maintiennent l'environnement contrôlé nécessaire à une fonction cérébrale optimale. Les deux barrières les plus notables sont la barrière hémato-encéphalique (BHE) et la barrière hémato-liquide céphalo-rachidienne (BHLCR) formée par les cellules épithéliales des plexus choroïdes. La grande majorité des molécules ne peut pas traverser ces barrières et seules quelques petites molécules liposolubles sont capables de pénétrer dans le cerveau passivement, et essentiellement toutes les macromolécules dont les protéines en sont exclues.

Ainsi, les besoins du cerveau à recevoir des éléments nutritifs et d'autres facteurs à partir du sang, et à éliminer les déchets métaboliques résultant de l'activité du cerveau sont satisfaits par des systèmes de transport très spécifiques. Ce mécanisme est connu sous le nom de transports médiés par un récepteur (RMT) impliquant la transcytose par des vésicules à travers la cellule. Ce mécanisme est dédié au transport des grosses molécules telles que des peptides et des protéines. Plusieurs de ces récepteurs ont été identifiés en tant que voies possibles d'accès au cerveau des molécules thérapeutiques pour traiter des maladies du système nerveux central mais sont encore en phase de développement.

L'objectif de ma thèse était d'essayer d'identifier de nouvelles voies pour la pénétration de molécules dans le cerveau. L'objectif à plus long terme étant de développer un véhicule capable de délivrer des médicaments pour traiter les maladies du système nerveux central.

Mon projet a mis l'accent sur la BHLCR pour identifier de nouvelles voies de transport médiés par les récepteurs en utilisant la technologie du « phage display in vivo ». Pour cette approche, une grande bibliothèque de bactériophages exprimant chacun à leur surface un anticorps spécifique a été utilisé. La bibliothèque est injectée dans la circulation sanguine périphérique et le LCR est ensuite échantillonné. Tout phage présent dans le LCR est récupéré et l'anticorps correspondant est identifié. En identifiant l'enrichissement de séquences spécifiques, nous espérons identifier des anticorps qui ont pu traverser la BCSFB en interagissant avec un récepteur médiant le transport. Cette approche ne fait
aucune hypothèse préalable sur la nature du récepteur ciblée et permet donc potentiellement la découverte de nouveaux systèmes de transport dans le cerveau.

Afin de valider les résultats obtenus in vivo et d'étudier plus en avant le transport des phages correspondant aux anticorps candidats, un modèle in vitro de la BHLCR a été établi en utilisant soit des cellules épithéliales primaires de rat issus de plexus choroïdes ou, plus commodément, une lignée cellulaire établie nommée Z310 (Dr. Zheng, Université de Perdue).

L'objectif de la première partie de la thèse est de développer un modèle in vivo pour étudier le transport du phage à travers la BHLCR. Pour cela, un certain nombre de phages de contrôles négatifs et de contrôles positifs ont été construits et produits. Les contrôles négatifs que nous avons préparés sont des phages «vides» exprimant seulement la protéine non modifiée gIII, ainsi que des phages recombinants qui expriment des anticorps non pertinents. Les contrôles positifs que nous avons construits sont des phages exprimant l’anticorps contre le récepteur de la transferrine du rat nommé OX-261, le facteur de croissance épidermique de rat (ligand), ou un anticorps à un domaine de la chaîne lourde (sdAb)2 FC5 qui a été identifié comme pouvant traverser la BHE3. Ces séquences d’anticorps ont été fusionnées au gène de phage gIII et sont donc exprimées sur la surface du phage. Les phages recombinants sont injectés dans la veine caudale du rat et on les laisse circuler dans le corps pendant un certain temps avant le prélèvement et l'analyse du LCR.

Une sélection des phages se liant au plexus choroïde a été effectuée en suivant le même protocole mais en utilisant une grande banque d'anticorps exprimés par des phages dans lesquelles les chaînes lourdes et légères d'anticorps sont exprimées sous forme d'un seul polypeptide séparé par un élément de liaison flexible. Dans ce cas, les phage non spécifiques sont éliminés lors du passage dans le système vasculaire périphérique. Le nombre «faux positifs» potentiels est donc diminué et il existe plus de chances d’identifier des récepteurs spécifiques pour les plexus choroïde. Les phages candidats intéressants sont ceux qui se lient à un récepteur de transport sur le plexus choroïde épithélium, et qui sont ensuite transportés dans le LCR. Ces phages sont susceptibles d’être rares, mais le système in vivo offre une sélection puissante pour les candidats de phages positifs.

Un challenge technique majeur, a été la difficulté à éliminer les traces de contamination sanguine lors de la collecte du LCR, puisque cela représente une source importante de
phage faux-positifs. Différents protocoles ont été testés et, nous avons finalement été en mesure de surmonter ce problème en utilisant un modèle de canulation à long terme de la « cisterna magna ». Plusieurs séries de sélection des phages in vivo ont permis d'identifier des candidats qui ont été ensuite reformatés en anticorps IgG normaux et caractérisés. Le but de la seconde partie de la thèse a été de mettre en œuvre un modèle in vitro de la BHLCR utilisant des cellules épithéliales primaires obtenues à partir du plexus choroïde de rat. La procédure a été adaptée à partir d'un protocole publié⁴. Dans ce modèle, les cellules sont ensemencées sur des membranes dans un système de culture à double chambre. Après 7 jours de culture, ces cellules montrent des propriétés biophysiques et biochimiques caractéristiques de l'épithélium in vivo. En parallèle, la lignée cellulaire épithéliale issue de choroïde plexus de rat Z310 a été utilisée comme une alternative aux cellules primaires. Les candidats ont été testés en utilisant ces deux modèles in vitro ainsi que dans une approche classique de sélection par liaison aux cellules (« binding biopanning »). Le modèle in vitro mimant le BCSFB a été mis en place et validé avec succès. Les candidats issus des expériences de sélection in vivo ont ensuite été testés mais malheureusement nous n'avions pas été en mesure de montrer un transport effectif de nos IgG reformatés à travers l'épithélium du plexus choroïde in vitro. Les raisons de ces résultats sont discutées.
INTRODUCTION

On one hand, the composition of the blood undergoes constant variation due to external factors such as diet, infection, composition of the atmosphere or physical activity. On the other hand, the correct functioning of the neurons requires a highly stable cerebral environment and thus the blood-CNS barriers have evolved to create a fluid milieu surrounding the brain cells which are thus protected from changes in serum composition. Barriers between the blood and the brain exist at several levels. The most highly studied is the so-called blood-brain barrier, or BBB, which surrounds the vast brain capillary network. Another major barrier exists between the blood and the cerebral spinal fluid (CSF) and is formed both by the epithelial cells of the choroid plexus (CP) which surrounds the four brain ventricles, and at the arachnoid membrane in the superficial compartment of the CSF. In addition a variety of other more specialized barriers have been identified including the, blood-retinal barrier, the blood-labyrinth barrier in the inner ear or blood-spinal cord barrier. The different fluid compartments of the brain and the characteristic features of each of the major barriers will be described in more detail below. In general terms, access to the brain is restricted through several different molecular mechanisms which recognise and regulate the entry of different classes of molecules.
A few low molecular weight (MW) lipid soluble compounds are able to diffuse passively from the blood into the brain, although most of these are rapidly ejected by an efficient efflux transport system which also eliminates unwanted metabolites produced as a result of normal brain metabolism. A series of specialized transporters and carrier proteins regulate the entry of essential nutrients such as glucose, nucleotides, amino acids, ions etc, while transport of larger molecules such as growth factors, Fe-transporters is regulated by specialized receptor systems in a process referred to as receptor mediated transport (RMT). These processes will be more fully described below. Clearly while such highly selective mechanisms are essential to maintain the constancy of the brain environment, they also restrict access of therapeutic molecules which could be useful in treating various CNS diseases. Thus much effort has been spent understanding each of these processes and investigating ways of bypassing, or exploiting, the various routes of entry into the brain. The approach described in the current thesis is an extension of the effort in pursuit of this goal.
1. Historical review of the concept of compartmentalisation between the blood and the CNS

If the CSF was first described around 1700BC\textsuperscript{6}, the notion of Blood Brain Barriers was first recorded in the XIX century. In 1885, Paul Ehrlich attempting to stain tissues \textit{in vivo} performed experiments in which he studied the distribution of a water soluble acidic dye following intraperitoneal injection. The results showed staining of all body tissues with the exception of the brain and the spinal cord\textsuperscript{7}. At this time he wrongly concluded that this was because the affinity of the dye for the CNS was low. Nevertheless, Paul Ehrlich was the first to propose the concept of drug targeting, the „magic bullet“ concept, for which he received the Nobel Prize in 1908. In 1913, his student Edwin Goldmann performed intracerebroventricular (ICV) injections of trypan blue dye\textsuperscript{8}, and observed that all the cells of the CNS were stained but that the peripheral organs were not, further suggesting the presence of a barrier between the CNS and the peripheral circulation. In 1913, Goldmann reproduced the experiment of Ehrlich by injecting the Trypan Blue by intravenous (IV) injection\textsuperscript{8} and confirmed the initial observations ().

![Figure 2: Representation of the experiment performed by Goldmann](image)

\textbf{Fig. A} : The Trypan blue dye is injected peripherally and do stain the peripheral organ but do not stain any organs of the CNS. \textbf{Fig. B} : The Trypan blue dye is injected directly into the brain and do stain the brain and the spinal cord but not the peripheral organ. Figure extract from Liddelow et al.\textsuperscript{6}
However, it was Lewandowsky\textsuperscript{9} in 1900, who was the first to propose a physical barrier between the blood and the brain and to use the term: “bluthirnschranke” or “blood brain barrier”.

Nevertheless, the idea of a barrier remained controversial, since in 1930 it was reported that some basic dyes injected in the bloodstream were able to stain the brain. Fridemann in 1942 and more precisely Davson in 1970 developed a theory which proposed that the passage from blood to brain or blood to CSF depends on a combination of size, charge and lipid solubility of the molecule\textsuperscript{10}. Davson also pointed out that some molecules were transported faster than expected\textsuperscript{6}. Thus it was in the 1970’s that the active transport of molecules between the blood and the brain started to be studied. These initial studies focussed mainly on the efflux of molecules from brain to blood, however, by the end of the 1980’s in order to meet the growing need for drug delivery in the treatment of CNS diseases, interest began to switch to the influx of molecules from blood to brain\textsuperscript{11}. In 2005, Pardridge\textsuperscript{12} reproduced the Goldmann experiment using modern techniques. He injected intravenously a small radiolabeled molecule (histamine) and he confirmed by autoradiography that the peripheral organs were stained but not the brain nor the spinal cord (\textsuperscript{13}).

![Autoradiography of adult mouse after IV injection of radiolabeled histamine](image)

\textbf{Figure 3: Autoradiography of adult mouse after IV injection of radiolabeled histamine}

Radiolabeled histamine was injected intravenously. Brain and spinal cord were not stained. Figure extracted from Pardridge et al.\textsuperscript{13}

As new data accumulated it became apparent that in fact there exist multiple barriers which protect the brain from the peripheral circulation. In this work, I will use the term blood–CNS barriers (BCNSBs) to include all of these barriers between the blood and the neural tissues of the CNS.
2. The diversity of Blood-CNS barriers

As mentioned above, there are at least three major and distinct anatomical structures. These are termed the Blood-Brain Barrier (BBB) which surrounds the cerebral blood capillary network; the Blood-Cerebrospinal Fluid Barrier (BCSFB) a layer of epithelial cells of the choroid plexus which restricts entry of molecules into the ventricular CSF and the Arachnoid membrane at the outer surface of the brain. In addition there is an internal membrane, the ependyma which regulates fluid exchange between the CSF and the brain interstitial fluid (ISF). In the adult brain, fluid exchange between these two compartments occurs more freely.

Each of these structures is shown in  and will be described in more detail below.
Figure 4: Main barriers and interface restricting fluid movement into and within the brain

**Fig. a:** Neurovascular unit showing endothelial cells (endo), tight junction (arrow), basement membrane (bm), astrocytes and pericytes (Peri); **Fig. b:** Blood-CSF barrier showing fenestrated endothelial cells, dashed arrows show the passage of molecules between the CSF and the blood vessel (bv), Choroid plexus epithelial cells (ep) with TJs (arrows); **Fig. c:** Arachnoid barrier from the meninges has TJ (arrows) in the outer cells of the arachnoid membrane (Arach) forming a barrier between the CSF-filled subarachnoid space (SAS) and the dura matter. **Fig. d:** Starp junctions (arrows) of the fetal neuroependyma in early development form a barrier. **Fig. e:** Mature neuroependyma is no more a barrier in adult (dotted arrows). Figure modified from Neuwelt et al.^{14}

### 2.1. The BBB

In the human adult brain, the total length of capillaries is approximately 600km with a surface area of the brain capillary endothelium estimated to be between 12m$^2$ and 20m$^2$^{5}.
Pardridge and Zlokovic\textsuperscript{17} have estimated that all cells in the brain are within 10-20μm of a capillary thus intriguingly, nearly every neuron in the human brain has its own individual blood supply. The network is so dense that molecules crossing the BBB have rapid access to almost the entire brain. Thus the trans-vascular route seems to be attractive in terms of therapeuic targeting of neurons.

**Neurovascular unit**

Until the 1990’s, the notion of BBB was used to define the brain capillary endothelial cells (BECs). In close proximity to BECs, pericytes, glial cells (especially astrocytes), neurons, together with the basal lamina (also called lamina basalis) ensheathing cerebral blood vessels, are indirectly involved in the establishment and maintenance of the BBB: these various cell types and basal lamina collectively constitute the ‘neurovascular unit’ (NVU) \cite{5}, a concept recently proposed to highlight the functional interactions which control BBB integrity.

![Figure 5: Neurovascular Unit structure](image)

Figure extracted from Abbott et al.\cite{5}. BL: Basal Lamina (or basement membrane)
Brain Endothelial Cells (BEC)

The endothelial cells which compose the vascular wall of the brain vessels are quite different from the endothelial cells of the peripheral capillaries. They are organized in a continuous monolayer and tightly sealed by the tight junction (TJ) proteins.

![Figure 6: Structure of Tight junction at the BBB level](image)

**Figure 6: Structure of Tight junction at the BBB level**

**Fig A:** EM of cross section through a brain capillary showing structure of the Tight Junction. Fig. extracted from Kristen et al.⁶. **Fig. B:** Representation of 2 BEC with the presence of TJ, AJ and JAMs protein. Fig. extracted from Abbott et al.⁵
TJ are the areas where two endothelial cells come together and where the junction is sealed by different families of specialized membrane proteins. The specific distribution of the junctional proteins results in a polarization of this zone of contact into the luminal 'tight junction' and the abluminal 'adherens junction' ( ). In addition to the proteins which span adjacent cells such as the JAMs, Claudins, Occludin, a complex network of intracellular regulatory proteins including cingulin, ZO-1, ZO-2, ZO-3, VE cadherin is also present. Tightness of the junction is regulated by the specific composition of the junction proteins and more rapidly by downstream events following receptor signalling.

The level of pinocytosis in BECs is very low compared to endothelial cells outside the brain which minimizes uptake of extracellular substances, while the lack of 'fenestration' (pores in the endothelial layer for rapid transport between blood and tissue) limits transcellular diffusion.

Astrocytes also contribute to the polarization of the endothelial membrane resulting in specific distribution of enzymes and receptors. The presence of specific enzymes at the BBB such as phase 1 oxidative enzymes like CYP2D6 implicated in the drug metabolism contributes to the metabolic barrier activity. The presence of efflux transporter at the basolateral side like P-glycoprotein transporter (P-gp) add a pharmacological barrier and results in oriented transporter-mediated transcytosis in which ligands are shuttled from one membrane to the other.

The NVU also allows limited movement of cells across the BBB. During development, some monocytes from the bone marrow enter the brain via the immature BBB and become resident immunologically-competent microglia. In adults, under normal conditions low-level infiltration of leukocytes can occur by diapedesis without changes in the TJ structure, while in response to certain pathological states, particularly those resulting in neuroinflammation, neutrophils are recruited into the brain via the paracellular pathway, albeit at a far lower level compared to other organs.

**Basement membrane and glial limitans**

The polarized endocytic cells which constitute blood capillaries are organized on a basement membrane (BM) composed of secreted extracellular matrix proteins. The BM of
the NVU is considerably more complex than that of the peripheral capillaries since it incorporates a second element, known as the glial limitans.

The BM functions to anchor the continuous endothelial cell layer by cell-matrix adhesion, and plays a major role in angiogenesis. The BM also contributes to the physical barrier which prevents entry of cells into the CNS compartment. The BM of the BBB is specialized compared to the BM from other locations.

**Pericytes**
Pericytes are embedded within the BM have phagocytotic activity which contributes to the barrier function. Pericytes are particularly important during development because the astrocytes are not yet present. By delivering molecules like plasminogen-activator inhibitor-1, they also participate in the endothelial homeostasis. Pericytes are also involved in the formation of the BM.

**Astrocytes**
The astrocytic end-feet are in direct contact with the BM and constitute an important additional component of the NVU known as the glial limitans. They express aquaporin 4, important in regulating the water content of the brain, as well as the Glial Fibrillary Acidic Protein (GFAP) which is useful as a biomarker for astrocyte labeling. Astrocytes are involved in the induction process during formation of TJs as demonstrated in vitro by the addition of astrocyte-conditioned medium which increases the TJ formation.

**Neurons**
Axons and neurites from the neurons are in contact with other actors from the NVU. Such neuronal projections do release several neurotransmitters to perivascular pericytes, endothelial cells or astrocytes participating to the regulation of the cerebral blood vessel. This field is not fully understand.
2.2. The BCSFB

Localisation and structure
The CSF flows continuously through the 4 brain ventricles, the spinal cord and the subarachnoid space where it exits the CNS via the saggital sinus (). It is actively secreted by the choroid plexus (CP) which is intimately associated with the lumen of each of the four ventricles. The direction of flow is from the 2 symmetrically located lateral ventricles (LV) through the 3rd and 4th ventricles and then via the cisterna magna into the spinal cord (see §3.2 for more details of the CSF circulation).

Figure 7: Localisation of the CPs within the brain
CP are located at the lateral ventricle, third ventricle and fourth ventricle (black arrow)

Each CP consists of a highly vascularized stroma surrounded by an epithelial layer which contacts the CSF and which is extensively convoluted to increase surface area (). The anatomical appearance of each CP is quite distinct: the CPs from the LVs are a more flat, leaf-like structure while the CPs from the 3V and especially the 4V are more complex and more highly lobed. Despite this, no major functional differences between the CPs from different locations have been observed.
**Fenestrated endothelial cell layer**

The highly vascularized stroma of the CP stroma contains a dense capillary network unlike that of the rest of the brain (Figure 8). The endothelial cells are fenestrated and lack TJs\(^{26}\) and do not present a major barrier to passage of molecules from the serum. These capillaries of the CP stroma have diameters of up to 15µm in contrast to the specialized brain capillaries of about 5µm diameter. The blood flow is thus much higher. The presence of gap junctions only\(^{27}\), makes possible a free exchange of molecules between the blood and the CP stroma by simple diffusion.

**CP Stroma**

The CP stroma is located between the CP endothelial cells and the CP epithelial cells. It is composed of fibroblasts, macrophages and granulocytes\(^{27}\) embedded in a complex extracellular matrix (ECM). Some stem cells are present in this ECM and have the possibility to differentiate into astrocytes or neurons\(^{28},^{29}\). Other specialized cell types present include the Kolmer cells\(^{30}\) and the epiplexus cells\(^{31}\).

![Figure 8: Localisation and structure of the CP from the LV](image)

*From Liddelow et al.\(^{32}\). LV: Lateral ventricle, CP: Choroid plexus, CPEC: Choroid plexus epithelial cells, BV: Blood vessel, St: Stroma, TJ: Tight junction*

**Choroid plexus epithelium (CPe)**

The key role of the BCSFB is the secretion of CSF. Indeed, as described in the §3.2 the CSF is not a simple filtrate from the plasma but has a specific composition and protein content. The distribution of the different transporters is polarized in the CPe. The CP
creates also an osmotic gradient between the blood and the CSF by the transport of chloride, sodium and bicarbonate. This gradient drives the secretion of water which is transported by water channels such as aquaporin. The secretion by the CP regulates the CSF composition and homeostasis: it also maintains the CSF pressure needed for suspension of the brain within the cranium which helps to protect it from external physical shocks.

The most important barrier function of the CP is provided by the CPe cell layer. It is highly convoluted and the apical surface area is estimated to be of the same order of magnitude as that of the BBB. The CPe cells have a cuboid form and associate into a monolayer which is sealed by TJ proteins.

CPE are highly polarized cells with asymmetrical distribution of the receptors. Transporters for leptin, BDNF, insulin, IGF-I, NGF, VEGF have been identified in both apical and basolateral membrane of the CPe allowing a bidirectional movement across the BCSFB. Mitochondria are abundant to provide energy needed for the secretory activities.

CPE express numerous TJ proteins (occludin, claudin-1, -2, -3, -6, -9, -11, -19 and -22) some of which such as claudin-1, -2, and -3 are enriched in the CPe and not highly expressed at the BBB. The presence of these TJs is responsible for the high TEER (transepithelial electrical resistance) of the CPe compared to other epithelia and for regulating paracellular transport of water-soluble molecules.

Regulation of the TJs has been more extensively studied at the BBB than at the BCSFB. It has been shown that the expression pattern of different TJ proteins plays a role in the 'tightness' of the intracellular contact. For example, claudin-2 and claudin-11 are expressed differently in the CP from the LV and from the 4V. The expression of TJs also differs during the development.

Cytoplasmic proteins associated with the ZO-1 are also expressed in the CPe. Other adhesion molecules are expressed in the CPe such as the Junctional Adhesion Molecules (JAM)-A, B and C or Endothelial Selective Adhesion Molecule (ESAM) or gap junctions proteins.

High metabolic activity of enzymes such as glutathione peroxidase or hydrolase are present at the CPe and act as an enzymatic barrier. The functional aspects of the barrier with the expression of specific transport pathways for the entry of specific molecules, and the numerous efflux transporters are described in the §6.
The ependyma

The ependyma is an extension of the CP epithelial cells which lines the cerebral ventricles and spinal canal. Cilia at the apical surface participate in circulating CSF. The ependyma is a barrier only during the early development. Its barrier function is greatly reduced in adult and it becomes permeable to the passage of all small molecules and many large proteins. This is not a blood-CNS barrier but separates the brain parenchyma from the CSF. The ependyma is sometimes referred to as the CSF–brain interface.

2.3. The arachnoid barrier

The Arachnoid barrier corresponds to the outer CSF-Brain Barrier (D). As shown in the , meninges are formed by 3 different layers: the pia and the arachnoid (forming the leptomeninges) and the dura.

Figure 9: Meninges view

Represent the Dura mater, the Arachnoid mater, and the Pia mater. Subarachnoid space is also represented. CSF filled the SAS and is reabsorbed in the superior sagittal sinus. Picture extracted from Antranik

The space between the arachnoid membrane and the pia mater is called the subarachnoid space (SAS). This cavity is filled with a trabecula tissue and the CSF. From the SAS the
CSF drains into the superior sagittal sinus for the re-absorption into the blood stream as described later (Figure 12).

### 2.4. Other barriers

Besides the 3 major barriers discussed above there are also other less well studied interfaces between the neural tissue and the blood\(^{42}\). Amongst these the blood-retinal barrier, the blood-nerve barrier, the blood-labyrinth barriers, the blood-spinal cord barrier have been described\(^{14}\).

The retinal vascular endothelium of the outer blood retinal barrier, the blood-nerve barrier (at the peripheral nerve), the blood-labyrinth barrier (limiting the cochlea) and the blood-spinal cord barrier (BSCB), each has a structure similar to the BBB with a continuous non-fenestrated endothelial cell layer sealed by TJs\(^{14, 43}\). The retinal pigment epithelium forms the inner blood-retinal barrier and has a structure similar to the BCSFB with an epithelium sealed by TJs\(^{44}\).

There is no anatomical nose-brain barrier but there is a nose-brain pathway where the olfactory bulb makes contact with the trigeminal nerves. This pathway involves extraneuronal transport and could be used for brain delivery as it bypasses the systemic circulation and does not come into contact with the BBB\(^{45}\).
The circumventricular organs (CVOs) are characterized by fenestrated capillaries and contain neural tissue. They are located in specific regions of the brain (Figure 10) and play a role in the neuroendocrine system. The absence of barrier function in the capillaries allows free exchange between blood and ISF. The classification of the CP as a CVO has been debated due to its fenestrated endothelium but it is not typically included due to the absence of neural tissue.

3. Description of the CNS fluids

Four main fluid compartments are present in the brain: the circulating peripheral blood, the internal milieu of the CNS known as the interstitial fluid (ISF), the cerebrospinal fluid (CSF) and the intracellular fluid of the cell which is regulated by the cell itself. As described above, the ISF and CSF are separated by the ependyma, however in the adult, the barrier function of this layer is weak and thus there is relatively free exchange between these two compartments.
3.1. Interstitial cerebral fluid (ISF)

Interstitial or extracellular fluid is located in the brain parenchyma and provides a regulated environment for the neurons and other brain cells. The volume of the ISF is about 225ml in human and the volume is carefully controlled by aquaporins notably aquaporin 4 present on the endothelium and astrocytic endfeet. The ISF represents about 20% of the total brain volume.

The ISF is derived in part from the CSF through the ependyma and with a minor exchange also occurring at the pia/glial layer in the SAS, and in part by secretion from the brain endothelial cells (BEC) which is driven by an apical Na\(^+\), K\(^+\), ATPase expressed at the abluminal side of the BEC. This ATPase creates an ionic and osmotic gradient leading to fluid uptake.

3.2. Cerebrospinal fluid (CSF)

The CSF is a clear liquid and the total volume of CSF in humans volume is about 140-150ml of which 25ml are contained in the ventricles and 125ml in the spinal chord and SAS. In the rat, calculations of total CSF volume range from about 90µl to about 250µl. As with the ISF, CSF secretion by the epithelium is driven by an apical Na\(^+\), K\(^+\), ATPase expressed on the apical membrane of the CPe. The CSF has a high turnover rate being completely replaced every 5-6 hours in both human and rat.

CSF is not a simple filtrate of the blood as it was thought at the beginning of the century, but a secreted fluid with tightly controlled and relatively stable composition containing proteins, amino acids, vitamins, ions, and a few cells. The protein concentration is low compared to blood (2-4mg/ml, or 0.5%-1% plasma protein concentration), but enriched in some specific proteins for example transthyretin. Abnormal accumulation of CSF causes an increase in the intracranial pressure, a condition known as hydrocephalus which can be lethal if untreated. It is mostly due to a problem in the CSF outflow (Figure 12).

In Human, CSF can be easily sampled and is frequently used to monitor diagnostic markers. Proteomics analysis in Human and in rat has been performed using lumbar puncture or cisterna magna puncture respectively. The CSF proteome has been shown to have diagnostic value. In Multiple Sclerosis, the presence of immunoglobulins in the CSF...
known as oligoclonal bands is an important diagnostic marker present in around 90% of all patients with multiple sclerosis\textsuperscript{65}.

![Figure 11: CSF flow through the human CNS](image)

Choroid plexus from the lateral ventricles (1) produces the CSF and flows to the choroid plexus from the third and fourth ventricle (2) where the CSF is enriched. Then the CSF flows to the spinal cord canal and the in the SAS (3). Reabsorption of the CSF into the blood stream occurs at the arachnoid villi (4). Figure extracted from Liddelow\textsuperscript{6}.

The CSF fills the brain ventricular system. As shown in the Figure 11, the CSF is secreted by the CP surrounding the LV, circulates through the foramen of Monro to the 3V where it is enriched by further secretion from the CPe, then through the aqueduct of Sylvius (also visible in the ) to the 4V where it is again enriched. CSF continues its flow into the foramina of Luschka and Magendie, the ventricular subarachnoid space, basal cisternae including the cisterna magna, and into the spinal cord, then returns via the SAS and the arachnoid villi in the sagital sinus. The arachnoid villi represent valves through which the most of the CSF is recycled back into the bloodstream (Figure 12).
Figure 12: Reabsorption of the CSF via the cranial sub arachnoid space (SAS)

The archnoid villi present at the SAS act as pressure-dependent valves for CSF absorption (Fig. B). Figure extracted from Sakka et al.51.

The main function of the CSF is to supply nutrient and essential molecules like amino acids or glucose to the brain and spinal cord by and to participate in maintaining CNS homeostasis. The rapid turnover of the CSF prevents accumulation of unwanted brain metabolites thus contributing to chemical stability of the CNS environment. This function is known as the “sink action” of the CSF52.

It also participates in neurohumoral brain modulation, neuroimmune interaction66 as well as the transport of hormones to the hypothalamus.

BCSFB and the CSF play a role in the immuno-surveillance within the CNS. It has been shown that BCSFB is a major point of entry for T lymphocytes and that the CP is able to initiate an immune response. The BCSFB is thus a link between the peripheral immune system and the CNS67.

4. BCSFB dysfunction in pathologies and ageing

There are many pathologies which have been shown to involve BBB dysfunction including stroke, trauma, infectious or inflammatory processes, multiple sclerosis, HIV, Alzheimer's disease, Parkinson's disease, epilepsy, brain and CP tumours, pain, glaucoma, lysosomal storage diseases (LSD)14. Similarly, many pathologies (neurodegenerative, inflammatory, infectious, traumatic, neoplastic, and systemic diseases) have also been linked to
dysfunction of the BCSFB which affects the permeability, transport or secretion of specific molecules into the CSF \(^{20,66}\).

In ageing, several structural changes are visible at the CP including atrophy of the CPe, fibrosis of the stroma or thickening of the basement membrane. All these changes lead to reduced secretion and reduced rate of CSF turnover\(^{68}\).

In Alzheimer disease (AD), the level of amyloid-beta (Aβ) is increased in the brain compared to normal brain, and according to the Aβ hypothesis, this is due to a reduction in the clearance of Aβ via different efflux pathways across the BBB\(^{69}\) or the BCSFB\(^{70}\). At the BBB the receptor for advanced glycation endproducts (RAGE), transports Aβ from the plasma to the ISF, while reverse transport from the ISF to plasma is mediated by the low density lipoprotein receptor-related protein 1 (LRP1) receptor\(^{71,72}\). Similarly at the CP, Aβ eflux is probably mediated by the LRP1 and LRP2 receptors\(^{73}\). CSF formation and turnover rates are also reduced in AD patients\(^{74}\) with changes in the CPe similar to those observed in ageing\(^{75,76}\).

In Multiple Sclerosis (MS) as well as in experimental autoimmune encephalomyelitis (EAE), the animal model of MS, it has been shown that the CP is the main entry of leukocyte into the brain \(^{66}\)\(^{20,77}\). In the healthy brain, the chemokine axis CCR6-CCL20 controls entry of lymphocytes at the CP and contributes to the immune surveillance of the CNS\(^{78}\). In the MS brain, inflammatory changes can be visualized by immunostraining. The presence of T lymphocytes in the vessels and stroma of the CP shows that CP represents a site of lymphocyte entry into the CSF\(^{79}\).

### 5. Problem of drug delivery: different strategies for accessing the brain

Pardridge has estimated that more than 98% of the small molecule drug candidates, and virtually all large molecules fail to cross the BBB\(^{80}\). Brain permeability requires the drug to be small (<400 daltons\(^{81}\)), have low net charge and be lipid soluble. Larger molecules such as peptides, proteins, antibodies or short interfering RNA (siRNA), are not able to cross the BCNSB and reach therapeutic concentrations without administering massive doses which would risk creating side effects on other organs. The percentage of the injected dose (%ID) is a parameter which reflects the relative distribution of an administered drug through different organs, and allows a comparison of the distribution between different tissues. This
parameter is expressed per gram of tissue (%ID/g). In the case of most antibodies, numerous reports indicate that the brain uptake is very low, less than 0.1%ID/g\(^1\). With such poor penetration, reaching a therapeutically relevant concentration of an antibody in the brain therefore requires experimental manipulation.

In view of the huge unmet medical need to treat CNS diseases, many different strategies have evolved to overcome the problems of low drug accessibility to the brain. Figure 13 gives an overview of the different approaches which will be described below. Essentially, the different approaches can be subdivided into invasive and non-invasive strategies.

![Figure 13: Overview of approaches to CNS drug delivery](image)

Adapted from Pathan et al.\(^8\) and Pardridge et al.\(^13\)

**5.1. Invasive strategies**

Neurosurgery can be used to bypass the BBB for local drug administration either by stereotactic injection or by use of implanted devices. According to the site of intervention this is called trans-cranial, intracerebroventricular (ICV) or intracerebral injection, intracerebral implant or intrathecal lumbar puncture\(^15\).
This approach has the advantage that drugs are injected directly into the CNS eliminating the problem of BCNSB impermeability and non-productive interactions with serum binding proteins and enzymes. Drug half-life is increased, and off-target side effects on other tissues are reduced\textsuperscript{83}. The obvious drawback of this strategy is that it requires delicate surgery, creates physical damage to the barrier with a risk of infection, and risk of damage to other brain areas\textsuperscript{83}.

Nevertheless, Gabathuler has reported several successful examples of intrathecal or ICV administration of drug and especially the continuous infusion of baclofen, a γ-Aminobutyric acid B (GABA-B) analogue used for the reduction of the spasticity in multiple sclerosis\textsuperscript{84,85}. This demonstrates that administration into CSF could have benefit for certain CNS conditions especially when the targeted receptor is located in the glands and other regions close to the ventricular system.

Another approach currently being investigated is transient disruption of the BBB using high-intensity focused ultrasound\textsuperscript{86} or manitol-induced osmotic shock\textsuperscript{87}. These 2 techniques have shown promising results however the repeated disruption of the BBB is not suitable for multiple administrations.

In conclusion, invasive strategies may have application in acute disease situations and possibly for brain cancers, but would not be appropriate in treatment of chronic CNS diseases.

\textbf{5.2. Non-invasive strategy as alternative route}

Clearly the peripheral vascular system provides the simplest route for drug delivery to access most of the internal organs and most drugs are developed for oral administration or direct injection. For the brain however, the vascular route has shown limited benefit, thus many non-invasive strategies have been proposed to facilitate the passage across the barrier. These include chemistry based strategies in which the drug-like properties of the molecule are optimized, or biology based strategies in which the drug is linked to a transporter molecule or incorporated into carrier systems such as nanoparticles, liposomes, polymers or micelles.
Another strategy consists of inhibiting the efflux transport however this is not without risk since the efflux transporters are usually not specific and are needed for the normal physiology of the brain. This is a difficult technique to control.

**Chemistry-based strategies**
The concept of the chemistry-based strategies is to modify the drug in order to meet the requirements of drug delivery into the brain. In practice, this often consists of modifying the liposolubility of the drug. This technique is useful for small molecules however, by increasing the liposolubility, there is often an increase in the size of the molecule which could create additional problems for the drug delivery, as well as a loss in potency.

**Biology-based strategies**
The aim of the biology based strategy is to use existing specific pathways to transport the drug. Depending on the drug to be transported this could target the carrier mediated transport (CMT) for small molecules or receptor mediated transport (RMT) for the larger molecules.

![Diagram of Brain Delivery Vector](image)

**Figure 14: Representation of a brain delivery vector**
Rv: Receptor for the vector. Adapted from Pardridge et al.80

Generally, chimeric molecules are constructed in which the drug is linked to a brain-selective transport vehicle often referred to as a ‘Molecular Trojan Horse’88.
The brain transport vector could be a natural or artificial ligand of a carrier or a receptor, or a peptidomimetic such as a monoclonal antibody (Mab) against an appropriate receptor. The used of natural ligands as vectors carries the risk associated with the activity of the ligand itself or induction of an immune response against it, and thus synthetic ligands or peptidomimetic antibodies are generally preferred. For antibody mediated delivery, a monoclonal antibody targets a receptor and is transported across the cell monolayer. Several peptidomimetic mAbs have been developed such as OX-26 and 8D3 which target the transferrin receptor and the human insulin receptor (HIRMAb) respectively (§6.4).

The brain delivery vehicle should target the complete transcytosis pathway and not only endocytosis, and strong receptor binding should be retained after the fusion with the drug\textsuperscript{81}. The barrier receptor should be highly expressed and if possible, the receptor should be localized specifically on the barrier to be targetted. While a strong receptor binding is clearly required, achieving maximum affinity to the receptor is not always desirable as was shown recently for the transferrin receptor\textsuperscript{89}. The high affinity antibodies generated initially bound the receptor but failed to release into the abluminal space and thus remained in close proximity to the capillary wall. To resolve this problem, the team at Genentech generated and tested lower-affinity antibodies against the transferrin receptor\textsuperscript{89} and this resulted in more effective release of the antibody from its receptor and as a consequence, increased diffusion of the drug into the brain parenchyma.

6. Transport pathways into the brain

A variety of different transport mechanisms have evolved to deliver ions, peptides, proteins or cells into the CNS. Transport can occur in either direction resulting in either influx or efflux. The different types of transporters are described in the Figure 15.
6.1. **Passive diffusion**

Passive diffusion across the CNS barriers can occur in the case of a few low molecular weight lipophilic molecules and drugs, however this is frequently counteracted by the presence of highly efficient efflux transporters such as organic acid transporter-3, peptide transporter-2 and and P-glycoprotein\textsuperscript{90}. Overcoming these challenges for drug delivery is one of the major goals of medicinal chemistry programmes which is beyond the scope of the current work and will not be discussed further.

6.2. **Paracellular pathway**

The paracellular pathway refers to the passage of cells or molecules between adjacent cells of the monolayer. This process is highly restricted at the BBB endothelium and the CPe epithelium due to the presence of TJs as described above (§0 and §0).

6.3. **Carrier mediated transporter (CMT)**

Carrier mediated transport provides a means of delivering some small molecules from the blood into the brain. Important examples are the LAT1 and glucose transporter 1 (GLUT1)
receptors which transport large neutral amino acids and glucose respectively\(^8\). Therapeutic use of large neutral amino acid transporter 1 (LAT1) has been made to deliver L-DOPA to the brain for the treatment of Parkinson’s disease.

### 6.4. Receptor mediated transcytosis (RMT)

Our interest has focussed mainly on RMT for the transport of large molecules such as proteins into the brain. Extensive studies have been carried out and a number of receptors and ligands have been identified. RMT involves vesicular trafficking across polarized cells and can be divided into 3 phases 1) receptor mediated endocytosis at the luminal surface, 2) movement of the vesicle through the cell cytoplasm and 3) exocytosis of the receptor-bound ligand at the abluminal surface of the cell\(^9\) (Figure 15).

The first application of RMT for drug delivery was described in 1984 for the use of the antibody OX-26 which targets the receptor of the iron transporter, transferrin\(^9\). \(^9\) 93. Subsequently the receptors for insulin\(^9\), leptin\(^9\) and tumor necrosis factor alpha\(^9\) were also investigated. Other ligands known to be transported by RMT include basic albumin, angiotensin II, insulin-like growth factor I and II and mannose 6 phosphate\(^8\). Table 1 describes the main receptor/ligand combinations to date which have been proposed as possible pathways for targeted drug delivery.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Natural Ligand(s)</th>
<th>Vector(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transferrin receptor (TfR)</td>
<td>Transferrin</td>
<td>OX-26 (antibody fragment)</td>
</tr>
<tr>
<td>LRP1</td>
<td>Melanotransferin (p97), receptor associated protein (RAP), amyloid precursor protein (APP), aprotinin, tissue factor pathways inhibitor and also Apolipoprotein E (apoE), a2 macroglobulin, tissue plasminoge activator (tPA), proteinase-inhibitors, Aβ, prion protein, aprotinin,...</td>
<td>p97 (protein), RAP (protein), Angiopep-2 (peptide)</td>
</tr>
<tr>
<td>LRP2</td>
<td>Insulin, insulin-like growth factor I (IGF-1), Aβ, RAP, Leptin, low density lipoprotein (LDL), thyroglobulin (Tg), apoE</td>
<td>RAP (protein), Angiopep-2 (peptide)</td>
</tr>
</tbody>
</table>
### Table 1: Main receptor and ligands used for drug delivery

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Ligand</th>
<th>Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin receptor (IR)</td>
<td>Insulin</td>
<td>Human IR mAb</td>
</tr>
<tr>
<td>a(2,3)-sialoglycoprotein receptor</td>
<td>a(2,3)-sialoglycoprotein</td>
<td>FC5 (antibody)</td>
</tr>
<tr>
<td>Diphteria toxin receptor</td>
<td>Diphteria toxin</td>
<td>CRM197</td>
</tr>
</tbody>
</table>

### 6.5. Choice of the positive control for RMT

For our project, we needed a positive control molecule in order to set up the conditions for our different *in vivo* and *in vitro* transport models across the BCSFB. Ideally this control would be an antibody since we plan to screen an antibody based library; it should target a receptor present only at the basolateral side of the CP and have restricted tissue distribution to achieve better specificity in our *in vivo* model. However to date, no clear candidates which meet these criteria have emerged from the literature. Thus based on previous work on the BBB and a knowledge of the receptors thought to be present at the BCSFB our initial experiments (Results §1) involved the construction and testing of a number potential positive control for this project.

All these candidates were cloned into a phagemid vector for expression at the surface of a phage and into a mammalian expression vector in order to produce the recombinant protein.

### 7. Phage display technologies

#### 7.1. Structure of a filamentous phage

Three closely-related filamentous phage have been extensively studied, f1, fd and the Ff group/class such as M13<sup>97</sup>. Monovalent M13 phage measures around 900nm in length, 9nm in diameter and has a molecular weight of about 15 million Daltons<sup>98</sup>. The capsid is composed of 5 phage-encoded coat proteins (G3p or GIIIp, G6p, G7p, G8p, G9p) which encapsulate the single stranded DNA genome of 6407 nucleotides in length. The G3p is the product of gene III and is present in 5 copies at the rounded end of the phage as seen in
the electron microscope. In most phage display technologies, the recombinant protein of interest is fused to the G3p protein and is thus displayed on the phage surface (Figure 16).

![Figure 16: Structure of a M13phage](image)

p8 is present in 2700 copies; G3p (or p3) present in 1-2 copies in helper phage and 5 copies in hyper phage

### 7.2. Biology of the filamentous phage

M13 phage infect the bacterial host though binding of the G3p protein to the bacterial F pilus. The binding causes a change in the phage structure which allows entry of the single-stranded DNA. The phage genome is then converted into double-stranded DNA for the formation of new single-stranded DNA molecules and transcription of the viral mRNAs which are then translated on the bacterial ribosomes. The single stranded DNAs are then packaged into virions and secreted. The complete infectious cycle takes about 10 minutes\(^9\). Filamentous phage follow a lysogenic cycle (i.e. are non-lytic) and do not kill the host.
7.3. The biopanning concept and general principles of the phage display approach

Phage selection, or panning, is usually performed using a library of recombinant phage particles each expressing a different polypeptide fused to the G3p protein (see below). A large library contains in excess of 1E9 different phage. Typically, panning is performed in vitro against an immobilized target bound to a plate or a tube. Non-bound phage are removed in different washing steps and the bound phage are eluted in various ways and amplified by reinfection of E. coli. Typically, several rounds of panning are performed and washing stringencies are increased in order to enrich for high affinity binders.\textsuperscript{100}
For the work described here we have adopted a similar approach but one in which the pannings were performed *in vivo* in the rat.

We have used an antibody library in which the variable regions of the 2 antibody chains (heavy and light) are expressed as a single polypeptide separated by a flexible linker. The linker allows the variable regions of each chain to assemble correctly to generate a functional antibody binding site. The recombinant antibody in this format is known as a single chain variable fragment (scFv).

To prepare recombinant phage, the scFv is cloned into a phagemid vector which is then transformed into a suitable E.coli host.
Recombinant phage are recovered or ‘rescued’ from the transformed E. coli by infection with a wild type (WT) ‘helper’ phage which provides all the coat proteins and machinery necessary to package the recombinant DNA into the new phage particle and release it into the bacterial growth medium from which it can be harvested and concentrated. When WT helper phage are used, most copies of the G3p protein are derived from the WT helper phage, while only 1-2 copies of the recombinant G3p are present. In a variant of the procedure which we have used in the later stages of this work, phage are rescued using a mutated helper phage in which gene III is deleted (so called ‘hyperphage’) and in this case, all copies of G3p expressed on the phage are derived from the phagemid and thus fused to the protein of interest. The reasons for this will be discussed in later sections.

**Other phage library formats**

Other phage library formats designed to exploit antibody diversity have been developed including libraries based on double stranded Fabs or more recently the single domain antibodies (sdAbs) present in camelid species which are composed of a single heavy chain variable region (V\_H) of only 12 to 15 kDa. Antibody diversity can also be built into various artificial scaffolds. A fuller description of these alternative formats are reviewed in Smolarek et al.\textsuperscript{101} and will not be described here.
7.4. Application of phage display to neurobiology

Several groups have used phage display in different formats to identify protein or peptide binders to the surface of brain cells. For most of this work selections are performed using brain cells in culture. Tanha et al. have identified two single-domain antibodies (sdAbs) FC5 and FC44, from a phage-displayed library of llama (sdAbs) following in vitro panning against human BECs\textsuperscript{102, 103}. Candidates were shown to transmigrate across endothelial cells in an \textit{in vitro} model of the BBB. This experiment shows that despite their large size, phage can be transported through a cellular CNS barrier.

Other studies have investigated phage internalization\textsuperscript{2, 97}. Using choroid plexus cells Gonzalez et al have shown internalization of phage displaying EGF through binding to the EGF receptor\textsuperscript{2}.

7.5. \textit{In vivo} phage display

\textit{In vivo} phage display selection is performed directly in a living organism and this has been most frequently used to find binders to specific organs or cell types. It has been used successfully to discover new biomarkers for a specific organ or disease. The main
advantage of using the *in vivo* route is that selections are performed directly *in situ* in the natural environment, thus there is no need to set up artificial binding and/or transport conditions.

Pasqualini\textsuperscript{104} using a peptide library was one of the first to adopt this approach to identify organ-selective binders to blood vessels of brain and kidney. Du et al.\textsuperscript{105} discovered a new biomarker for hepatocarcinoma and Giordano et al. by combining *in vitro* and *in vivo* selection identified peptides which bind specifically to the lung vasculature\textsuperscript{106}. Wan et al. have performed an *in vivo* selection using a phage peptide library which was administrated intranasally. Phage were recovered in the brain and a peptide (ACTTPHAWLCG) was identified which translocated 50 fold more efficiently than a non specific peptide\textsuperscript{107}.

8. Our approach

8.1. *In vivo* phage display to investigate transport across the BCSFB

We have used *in vivo* phage display in rats to screen for antibodies capable of delivering a cargo from the periphery into the CSF. In our approach, a library comprising $2.8 \times 10^9$ independent phage is injected into the peripheral circulation of several rats and after an appropriate time interval the CSF is collected. Any phage which have entered the CSF are recovered and can be amplified in E. coli for re-injection into fresh rats. After multiple rounds of *in vivo* panning, phage-encoded antibody fragments (scFvs) recovered from the CSF are sequenced and any enrichment for specific molecules would be indicative of an antibody-mediated transport process from the peripheral circulation into the brain.
The phage-encoded scFvs would then be characterized further as possible vehicles for transporting other molecules into the CSF. For this the scFvs are reformatted as normal IgGs and their vector properties can be explored by fusion to a suitable reporter sequence such as green fluorescent protein (GFP) or LacZ. Injection of the purified reporter fusion protein into rats would allow the transport of the reporter sequence to be tracked through the CSF and brain parenchyma. This would simulate conditions in which the antibody-bound tracker could be a potential therapeutic cargo.

Analysing the identity, distribution and properties of the antibody target protein(s) would constitute an interesting launching point for further study and clinical development.

8.2. Rationale for studying the BCSFB

The CSF in Human occupies a volume of around 170ml, and is continually secreted into the brain ventricular system by the CP. Estimates of the bulk flow suggest that it is completely replaced approximately every 6h. While some of the molecules which enter the CSF are synthesized within the CP, many are transported directly from the blood thus we predict that many and varied pathways must exist to transport molecules from the highly vascularized CP stroma across the CP epithelium and into the CSF. In addition, the transport systems in the BCSFB have been relatively unexplored compared to the BBB, which thus offers greater opportunity for identifying novel routes into the CNS.

A further advantage for transport studies across the BCSFB is that the fluids on both sides of the barrier, serum and CSF, can be sampled directly. In contrast, assessing transport across the BBB is complicated by the difficulty of obtaining the brain ISF without first
disrupting the brain tissue and thus introducing inherent uncertainty about contamination by non-brain fluids. The ability to sample the CSF directly thus allows us to perform not just binding studies but functional assays in which the readout is for fully transported molecules. We have opted to search for transported molecules using *in vivo* phage display, even though the unknown issue is whether it is possible to transport something as large as a phage particle using natural pathways. However in this respect, the BCSFB may be a more amenable barrier in that it consists of a single cell layer, the CPe, while the BBB is a more complex structure involving both the endothelial barrier and the astrocytic endfoot/glial limitans as described above (§0).

The simpler BCSFB structure also facilitates *in vitro* testing of transported molecules through cell monolayers in culture. The CP is a well defined anatomical structure which can be dissected cleanly, and methods have been developed to culture the CPe cells as fully confluent monolayers on transwell membranes in which their barrier function can be monitored by measuring their electrical properties (see below). This therefore provides an independent approach to validating any positive candidates from our *in vivo* screening.

In the longer term, one issue with targeting the CSF as a means of introducing therapeutic molecules to treat brain disorders could be that the flow rate of the CSF through the brain ventricular system may limit the accessibility of the therapeutic to the brain tissue. On the other hand, as described above (§5.1) ICV injection has proved considerably more efficient than IV injection for many CNS applications and this is a useful paradigm in evaluating our longer term strategy.

### 8.3. *In vitro* model transport of the BCSFB

As mentioned above, the CP can be dissected cleanly and relatively easily even from the embryonic rat brain. Each of the 4 brain ventricles has an associated CP although the morphologies are quite distinct. Generally the CP associated with the 2 lateral ventricles are handled separately from the CPs of the 3rd and 4th ventricles.

Several groups have developed methods for purifying and culturing primary CPe cells either in culture dishes or on plastic support membranes. The latter can be adapted for dual chamber cultures giving access to both the lower (basolateral) and upper (apical) surfaces suitable for transport studies. Cells are seeded at high density and as the cultures become...
confluent, TJ are formed and can be monitored by assessing the trans epithelial electrical resistance (TEER) or the cell capacitance (see below). The main difficulty in working with primary cells is that cultures can only be successfully established using embryonic brains, and thus the number of cells available is limited. To overcome this limitation, different groups have attempted to develop cell lines from the choroid plexus epithelium. We have acquired 2 cell lines derived from rat CPe: Z310\textsuperscript{108-110} and TR-CSFB\textsuperscript{111, 112}.

8.4. Characterization of the \textit{in vitro} model

In order to validate the CP \textit{in vitro} model, a series of tests are performed. The cell morphology should be cuboid and not elongated which in primary cell cultures would indicate contamination with fibroblasts. By electron microscopy, microvilli should be visible on the apical surface. A convenient molecular marker for CPe cells is transthyretin (TTR) also known as prealbumin which is synthetised specifically in the CPe and transports thyroxine into the CSF\textsuperscript{113}.

The specific pattern of TJ proteins expression can also be checked once cells reach confluence and the electrical resistance, or TEER, provides a good functional characterization of the barrier 'tightness'. TEER is explained in detail in the Material and methods section §5.2.4. Finally, visual inspection of the cultures should reveal a difference in medium level between the two chambers with a higher level in the upper chamber due to the flux across CPe which simulates production of the CSF.
MATERIAL AND METHODS

1. Molecular biology

1.1. Material for molecular biology

The following products were used: RNAqueous kit (Ambion, #AM1912); absolute ethanol *pro analyse* (Merck, #1.00983.1000); SuperScript III kit (Invitrogen, #18080-051); Pwo Master kit (Roche, #03 789 403 001); AmpliTaq Polymerase (Applied Biosystem, #4398808); ethidium bromide (Invitrogen, #155-85-11); loading buffer (5X) (Bio-Rad, #161-0767); DNA Molecular Weight Markers 100bp ladder (Roche, # 11721933001); rAPid Alkaline Phosphatase kit (Roche, #04 898 133 011); MAX Efficiency® DH10B™ Competent Cells (Stratagene); Wizard Plus SV Minipreps DNA purification system (Promega, #A1330); Big Dye Terminator (BDT) (Applied Biosystem, #4337455); BDT Buffer 5X (Applied Biosystem, #4336701); Sephadex G50 column (GE-Healthcare, #17-0573-02); ultra Pure Agarose powder (Invitrogen, #16500-500); restriction enzyme and buffer were from New England Biolabs, T4 DNA ligase (New England Biolabs, # M0202S); Transcriptor cDNA kit (Roche, #05-081-955-001); pBluescript II SK- Phagemid Vector (Agilent/Stratagene, #212206); λ DNA-HindIII Digest ladder (New England Biolabs, #N3012S); Bromophenol Blue (Sigma, #B0126-25G); Ampicillin (Sigma, #A1593); MAX Efficiency® DH5α™ Competent Cells (Life technologies, #18258-012); ElectroMAX™ DH10B™ Cells (Life technologies, #18290-015).

The following items of equipment were used: horizontal electrophoresis system (Wide Mini-Sub Cell GT System, Bio-Rad, #170-4405); DC power supply (Bio-Rad #165-5050); NanoDrop 1000 spectrophotometer (Thermo Scientific, #ND1000) with its Nanodrop software; PCR Sprint Thermal cycler (Thermofisher, #HB-SP-05-220/110); electroporation cuvette (0.1cm) (Bio-Rad, #165-2089); electroporator system Gene-Pulser (Bio-Rad, #165-2105); Qiagen BioRobot 8000 (Qiagen, # 81110010); adhesive PCR Film (Thermo Scientific, #AB-0558); 3700 DNA sequencer (Applied Biosystems, #3700).

The following mediums were prepared by our facilities with the following composition:
Lysogeny broth (LB) agar plate with 10g/l tryptone (Merck, #1.07213), 5g/l yeast extract (Merck, #1.03753 5), 10g/l sodium chloride (NaCl) (Merck, #1.06404) and agar powder (Merck, #1.01614) adjusted to pH=7 with 10M sodium hydroxide (NaOH).
Super Optimal broth with Catabolite repression (SOC) with 2% w/v tryptone, 0.5% w/v Yeast extract, 10mM NaCl, 2.5mM KCl, 10mM MgCl₂ and 20mM glucose diluted in water.
Terrific broth medium with 1.2% peptone, 2.4% yeast extract, 72 mM K₂HPO₄, 17 mM KH₂PO₄ and 0.4% glycerol.

1.2. Method of cDNA production by RT-PCR
The genes of most of the control proteins used in this study (eg leptin, IGF and LRP2) were cloned by RT-PCR. This process consists in first making a cDNA copy of the mRNA using the reverse transcriptase (RT) and then amplifying the coding sequence as double stranded DNA using the polymerase chain reaction (PCR) in the presence of specific oligonucleotide primers. Primers generally include restriction endonuclease recognition sites at the 5’ end to facilitate subsequent cloning into a plasmid vector.

1.2.1. RNA isolation
All work was performed in a RNase-free environment (tubes, bench, pipettes, gloves, etc). The RNAqueous kit was used to isolate the total RNA according to the protocol provided by the manufacturer. Cells or tissues were disrupted in lysis buffer containing guanidinium thiocyanate and diluted with absolute ethanol to a final concentration of 66% v/v. The lysate/ethanol mixture was transferred into a spin-column containing silica resin which retains RNA bound to the resin surface while other materials (protein and DNA) are removed from the column by centrifugation. After several washing steps with 70% ethanol the column was air-dried and the RNA was eluted with RNase-free water preheated to 75°C. The RNA concentration was then measured using the Nanodrop spectrophotometer and an aliquot of the RNA sample was used for RT-PCR.

1.2.2. Reverse Transcription
The SuperScript III kit was used. A reaction mixture was prepared according to Table 2 containing the isolated mRNA, the nucleotide triphosphates and an oligonucleotide primer.
of 20-25 nucleotides complementary to a region of the mRNA downstream of the region to be cloned. The solution was incubated for 5min at 65°C to denature the RNA and placed on ice for at least 1min. The cDNA Synthesis solution was prepared in parallel. Both solutions were mixed (vol 1:1) and incubated sequentially for 50min at 50°C, 5min at 85 °C and 1min on ice. A volume of 1µl of RNase H (provided within the kit) was added and the incubation continued for a further 20min at 37°C to remove the RNA strand from the RNA-cDNA heterodimer.

<table>
<thead>
<tr>
<th>RNA/primer solution</th>
<th>cDNA Synthesis solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>6µl of DEPC-treated water</td>
<td>2µl Reverse Transcriptase buffer (10X)</td>
</tr>
<tr>
<td>1µl of primer (50 µM)</td>
<td>4µl of MgCl₂ (25mM)</td>
</tr>
<tr>
<td>1µl of dNTP mix (10mM)</td>
<td>2µl of DTT (0.1M)</td>
</tr>
<tr>
<td>2µl of total RNA (0.5mg/ml)</td>
<td>1µl of RNaseOUT (40U/µl)</td>
</tr>
<tr>
<td></td>
<td>1µl of SuperScript III RT (200 U/µl)</td>
</tr>
</tbody>
</table>

Table 2: Preparation of the solutions needed for the reverse transcriptase reaction

For longer cDNAs, the RT step was performed using Transcriptor cDNA kit following the protocol from Roche.

1.2.3. **Polymerase Chain Reaction**

Polymerase Chain Reaction (PCR) was used to amplify the DNA sequence of interest. Different polymerases were used depending on the experimental design.

**Amplitaq Polymerase**

The following mix was prepared: 1X AmpliTaq Buffer supplied 2mM Magnesium Chloride, 200µM of each deoxiribonucleotide (dNTP), 0.1µg of DNA, 0.5µM forward primer, 0.5µM reverse primer, PCR-grade water up to 100µl and 2.5 units of AmpliTaq DNA Polymerase. The PCR reaction was performed in 0.2ml microtubes. The Taq polymerase was added at a temperature superior to 85°C to minimize amplification of primer dimers. The temperature of the annealing step and the time of extension were varied depending on the melting temperature (Tm) of the primers and the size of the fragment to be amplified. In general the annealing was performed at 5°C below the Tm of the primers, and the extension time was
calculated allowing 1min for every 1000 nucleotides (Table 3). PCR reactions were performed with a PCR thermal cycler.

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
<th>Stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>94°C</td>
<td>3min</td>
<td>Holding</td>
</tr>
<tr>
<td>Denature</td>
<td>95°C</td>
<td>30sec</td>
<td>Cycling</td>
</tr>
<tr>
<td>Anneal</td>
<td>50°C-65°C depending on primer Tm</td>
<td>30sec</td>
<td>(25 to 40)</td>
</tr>
<tr>
<td>Extend</td>
<td>72°C</td>
<td>60sec/kb</td>
<td></td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C</td>
<td>7min</td>
<td>Holding</td>
</tr>
<tr>
<td>Final hold</td>
<td>4°C</td>
<td>For ever</td>
<td>Holding</td>
</tr>
</tbody>
</table>

Table 3: PCR Program used for AmpliTaq

Pwo PCR kit contains a proof reading activity and was preferentially used to perform an accurate amplification of fragments up to 3kb. A solution containing 50ng DNA template, 0.5µM forward primer, 0.5µM reverse primer in a final volume of 40µl was mixed 1:1 on ice with the Pwo master mix and subjected to thermocycling as follows:

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
<th>Stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>94°C</td>
<td>2min</td>
<td>Holding</td>
</tr>
<tr>
<td>Denature</td>
<td>95°C</td>
<td>15sec</td>
<td>Cycling (25 to 40)</td>
</tr>
<tr>
<td>Anneal</td>
<td>Primer Tm</td>
<td>30sec</td>
<td></td>
</tr>
<tr>
<td>Extend</td>
<td>72°C</td>
<td>45sec/kb</td>
<td></td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C</td>
<td>7min</td>
<td>Holding</td>
</tr>
<tr>
<td>Final hold</td>
<td>4°C</td>
<td>For ever</td>
<td>Holding</td>
</tr>
</tbody>
</table>

Table 4: PCR Program used for Pwo

1.2.4. **Agarose gel electrophoresis**

DNA fragments were visualized using a horizontal electrophoresis system in the presence of ethidium bromide. The percentage of agarose needed for the gel varies with the size of the band to visualize (Table 5).
<table>
<thead>
<tr>
<th>Recommended percentage of agarose</th>
<th>Optimim resolution for linear DNA (in bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>1000-30000</td>
</tr>
<tr>
<td>0.7</td>
<td>800-12000</td>
</tr>
<tr>
<td>1</td>
<td>500-10000</td>
</tr>
<tr>
<td>1.2</td>
<td>400-7000</td>
</tr>
<tr>
<td>1.5</td>
<td>200-3000</td>
</tr>
<tr>
<td>2</td>
<td>50-2000</td>
</tr>
</tbody>
</table>

Table 5: Recommended percentage of agarose versus DNA size

Table adapted from the Promega recommendation

This common technique was used for multiple purposes throughout this work such as validation of a PCR product, the control of fragment purity, purification of a band of interest (by cutting the band out of the gel), control for ligation or cleavage by restriction enzyme. To obtain the required percentage gel, ultra Pure Agarose powder was added to the tris acetate EDTA (TAE) base buffer and heated in a microwave oven to just below ebullition. An appropriate comb was installed on the gel-casting system and the gel was cast avoiding air bubbles. Polymerization was performed at room temperature (RT) for about 1h, and then the gel was transferred into the running system. Ethidium bromine was added to a final concentration of 250ng/ml in the running buffer (TAE 1X). The power supply was set-up at 300mA and the gel was pre-run for 30min.

Typically, 6µl of a PCR reaction was diluted with 2 volumes of water to reduce the salt concentration, and 4µl loading buffer (5X) containing Bromophenol blue was added prior to loading. Samples were electrophoresed at 300mA until the dye reached the end of the gel. DNA Molecular Weight Markers (Lambda/HindIII or 100bp ladder) were included as appropriate. The DNA bands were visualized using a UV transilluminator and the digital image was recorded for further analysis. For preparative electrophoresis up to 250ng of DNA was analyzed by agarose electrophoresis and the band of interest was excised under UV illumination and purified from the gel as described below.

1.2.5. DNA Clean-up

The DNA clean up is a step used either after PCR to remove contaminating primers, nucleotides and enzymes, or to remove the agarose from DNA fragments after electrophoretic purification. The protocol provided by the manufacturer (Wizard® SV Gel and PCR Clean-Up System) was used. PCR products were diluted 1:1 with Membrane
Binding Solution; gel purified DNA bands were diluted with Membrane Binding Solution (10µl per mg of agarose) and heated to 55°C until complete dissolution of the gel. The sample was then transferred to the spin-column provided and incubated for 1 min at RT to ensure the binding of the DNA. After centrifugation, the flow-through was discarded and the column was washed twice with the Membrane Wash Solution. The column was allowed to dry completely and 50µl nuclease free water was added and left for 1 min. The column was then centrifuged with a new collecting tube to recover the eluted DNA. Once purified, the DNA can be used directly for sequencing, digestion or other downstream procedures as needed.

1.2.6. **Determination of nucleotide concentration**

Total DNA or total RNA concentration was determined using the NanoDrop 1000 spectrophotometer which enables sensitive and accurate measurement using only 2µl sample. Optical density (OD) was measured at 260nm given that OD$_{260}$=1.00 AU (absorbance unit) corresponds to 50µg/ml double stranded DNA or 40µg/ml RNA. The purity of the nucleotide sample was estimated by measuring the OD$_{280}$ which reflects the protein contamination. A ratio of 260nm/280nm of >1.8 was considered acceptable. Nanodrop could be also used to measure protein concentration.

![Figure 22: Nanodrop spectrophotometer equipment](image)

This equipment allows the measurement of the absorbance at different wavelengths in a small volume sample (2µl). Figure provided by the supplier Thermo scientific.
1.3. Method of cDNA cloning into plasmid vectors

For expression of recombinant protein, the cDNA is subcloned into an appropriate expression vector. In this study a vector known as pTT5, which is particularly well suited for protein expression in HEK293 cells, has been used for most experiments. cDNA subcloning involved several steps. First, the cloning vector and the expression host were chosen. The cDNA is amplified by PCR using primers designed to insert appropriate restriction sites flanking the fragment to be cloned. Next, the insert and vector were digested using the selected restriction enzymes (§1.3.1) and gel purified. The ligation was performed (§1.3.3) and the ligated products were used to transformed E.coli using the plasmid encoded antibiotic resistance gene for selection of successfully transformed bacteria (§1.3.4). The plasmid DNA was extracted from individual transformed bacterial colonies (§1.3.5), and the DNA was sequenced (§1.3.6), in order to confirm the correct construction (§1.3.7).

1.3.1. **Enzymatic digestion**

PCR products with terminal recognition sites for restriction enzymes were generated by PCR and purified as described above, digested using the appropriate restriction enzymes for 2h-16h at 37°C. The choice of the buffer depends on the restriction enzyme chosen. The vector and the insert are usually digested with the same enzymes. After digestion, the digested insert and linearized vector are purified (§1.2.5).

1.3.2. **Deposphorylation of the vector**

Deposphorylation of the vector was sometimes performed to prevent vector recircularization during ligation, thus increasing the frequency of positive ligation products. In practice this was mostly used for subcloning with a single restriction enzyme when both ends of the linearized vector had compatible (ligatable) ends. For these experiments the rAPid Alkaline Phosphatase kit was used. The following mix was prepared: 30µl of DNA vector, 5µl of rAPid Alkaline Phosphatase Buffer 10X, 2.5µl of rAPid Alkaline phosphatase, 2.5µl of PCR-Grade water, for a total of 40µl. The solution was mixed and centrifuged briefly and incubated for 30min at 37°C to remove the phosphate from the 5’ end. The solution of rAPid Alkaline Phosphatase was
then inactivated by increasing the temperature to 75°C for 2min. The dephosphorylated vector was run on a 0.8% agarose gel and the band of interest was isolated and cleaned using Wizard® SV Gel and PCR Clean-Up System (§1.2.5). Concentration of the cleaned, digested, dephosphorylated vector was then measured (§1.2.6) and the vector was aliquoted and frozen at -20°C for further use.

1.3.3. **Ligation**

For ligation, the digested and purified vector and insert DNAs were mixed in a molar ration of 1:3 (vector:insert) and incubated in the presence of 1X ligation buffer and 1 unit of T4 DNA ligase for 1h at RT. To calculate the amount of insert needed for ligation (Qi), the following formula was used: 

$$ Qi = R \times \frac{Li}{Lv} \times Qv $$

where Qv is Quantity of Vector (usually about 10-20 ng), Li is Length of the Insert (bp), Lv is Length of the Vector (bp), and R is the Ratio = 3. An aliquot of this ligation product was used to transform a recA- E.coli strain such as DH10B.

1.3.4. **Transformation by electroporation**

LB-agar transformation plates were prepared containing 100µg/ml ampicillin and dried under a laminar flow hood for 30min. For each plate, 10µl 0.4M Isopropyl β-D-1-thiogalactopyranoside (IPTG) transcriptional inducer, 40µl 2% X-Gal chromogenic substrate (made up in dimethylformamide), and 150µl of water were mixed and added in the middle of the plate and spread homogeneously across the surface with a spreader.

1µl of the ligation product and 40µl of the diluted E. coli DH10B electrocompetent cells were mixed in the bottom of the electroporation cuvette (0.1cm) and chilled on ice. The electroporator system Gene-Pulser (Figure 23) was set to 1.7kV according to the Bio-Rad protocol for a 0.1cm cuvette. The lid was closed and the electroporator was activated. Immediately, 1ml pre-warmed SOC medium was added and the cells were transferred to a 10ml tube, and incubated for 1h at 37°C under agitation. The transformation mixture was then transferred in a 1.5ml microtube and centrifuged at 6000rpm for 3min at RT. The supernatant was removed and the bacterial pellet was resuspended in 100µl of SOC medium and spread on the LB-Amp transformation plate. The plate was incubated overnight at 37°C or during the week-end at RT. White colonies were selected for further analysis.
1.3.5. DNA plasmid preparation

For only a few samples, the preparation was done manually as described: a total of 5ml of 2YTAG or LB medium were inoculated with a single bacterial colony. The inoculated medium was incubated overnight at 37°C under agitation. The next day, an aliquot of the overnight culture was removed to create a glycerol stock, and plasmid DNA purification was performed using the Wizard Plus SV Minipreps DNA purification system according to protocol provided.

For a 96 well plate, the Qiagen BioRobot 8000 was used: A volume of 1.5ml of complete terrific broth medium (TB) containing 1X Phosphate Buffer 10X, 2% Glucose, 100µg/ml ampicillin medium was dispensed per well in a 96 deep well plate, and an isolated colony was manually picked with a toothpick into each well. After 5min, the toothpick was removed carefully. The cultures were incubated overnight at 30°C (750rpm). An aliquot of the overnight culture was kept to create a glycerol stock. The 96-well plate was centrifuged at 4000rpm for 10min and the supernatant was discarded. The 96-well plate containing the pelleted bacteria was put into the miniprep robot and DNA extraction was performed according to the Qiagen protocol. DNA concentration was checked by measuring the OD$_{260}$ (§1.2.6).
Up to 20µg plasmid DNA were obtained from 5ml to 20ml of bacterial culture. For larger scale preparations midiprep (Qiagen, #12943), maxiprep (Qiagen, #12963), megaprep (Qiagen, #12981) or gigaprep (Qiagen, #12991) were used to yield approximately 250µg, 1mg, 2.5mg and 10mg respectively.

1.3.6. DNA sequencing using the DNA sequencer robotic system

Preparation of the PCR solution: 84µl Big Dye Terminator (BDT) + 366µl distilled water + 120µl of primer at 5pmol/l (or 5µM) + 150µl BDT Buffer 5X. 2µl of plasmid DNA (250-500ng) were added and 6µl of the PCR solution were dispensed in the PCR 96 well plate. The plate was sealed with an adhesive PCR film and centrifuged for 1min at RT before performing the PCR (cf§1.2.3).

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>96°C</td>
<td>2min</td>
</tr>
<tr>
<td>96°C</td>
<td>15sec</td>
</tr>
<tr>
<td>55°C</td>
<td>5sec</td>
</tr>
<tr>
<td>60°C</td>
<td>30sec</td>
</tr>
<tr>
<td>60°C</td>
<td>4min</td>
</tr>
<tr>
<td>4°C</td>
<td>For ever</td>
</tr>
</tbody>
</table>

Table 6: PCR program for sequencing

PCR products were purified on a Sephadex G50 column and analyzed on the DNA sequencer according to the manufacturer's protocol.

1.3.7. Sequences analysis

Multiple alignments and identification of complementarity determining regions (CDRs) in antibodies were performed using an open access database of germ-line variable genes from the immunoglobulin loci called Vbase 2 (Retter I, Althaus HH, Münch R, Müller W: VBASE2, an integrative V gene database. Nucleic Acids Res. 2005 Jan 1;33(Database issue):D671-4.) For DNA analysis, Seqman Pro and Edit Seq software from DNA Star Lasergene (version 8.1.2) were used. For virtual cloning and annotated map of DNA, Clone
manager Professional Suite (version 8) was used. For all the other bioinformatic tools (alignment, annotation, database, structure, sequence,..), internal programs were used through our RUMBA interface.

1.4. Detail of the different clonings of OX-26

DNAs encoding the $V_H$ and $V_L$ were amplified and cloned first into the BSK cloning vector (§1.4.1). To express recombinant OX-26, DNA was then subsequently reformatted into the pTT expression vector (§1.4.2). To express the scFv OX-26 phage for use as a control for *in vivo* studies, DNA was reformatted into pHal14 phagemid vector (§1.4.3).

1.4.1. Cloning of VL OX-26 and VH OX-26 from OX-26 hybridoma cells into pBluescript vector

Analysis and design of the degenerate primers for OX-26 variable regions

In order to obtain the specific cDNAs corresponding to the light and heavy chain from the OX-26 antibody, degenerate PCR primers were designed based on the results in Table 7. For the light chain, 4 degenerate primers covering a total of 960 possible 5' sequences were designed; for the heavy chain 2 degenerate primers covering a total of 641 possible 5' sequences were designed. BamHI restriction sites were added at the beginning of each primer. The bottom strand primers were designed based on the invariant constant regions of the light or heavy chain. Primer sequences are given in the appendix part.

RNA Isolation

RNA extraction from 1E8 cells was performed and the total RNA concentration was measured with the Nanodrop at 166ng/µl and 15µl was used to analyse the integrity of the RNA by electrophoresis on agarose gel as shown in the Figure 24.
Figure 24: Quality of RNA checked on 1.2% agarose gel

The presence of intact 18S and the 28S rRNA bands confirms the integrity of the RNA.

**RT-PCR**

The reverse transcriptase step was performed using either oligo-dT or random hexamer primers. The PCR step was performed using the primer pairs described in the Table 7. The 4 PCR reactions for the LC and 2 PCR reactions for the HC to cover all the coding possibilities. Two series of PCR reactions were performed using the cDNAs from either the oligo-dT or the random primers.

<table>
<thead>
<tr>
<th>LC OX-26</th>
<th></th>
<th>HC OX-26</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Top strand</td>
<td>Bottom strand</td>
<td>Top strand</td>
<td>Bottom strand</td>
</tr>
<tr>
<td>MS1188 (288)</td>
<td>MS1192 (1)</td>
<td>MS1193 (384)</td>
<td>MS1195 (1)</td>
</tr>
<tr>
<td>MS1189 (288)</td>
<td></td>
<td>MS1194 (256)</td>
<td></td>
</tr>
<tr>
<td>MS1190 (192)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MS1191 (192)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Table 7: Primers used to PCR out the OX-26 sequence*

Bottom strand was designed to recognize the invariant mlgG constant region. The numbers in brackets correspond to the redundancies for each primer set. All the primers include a 5' BamHI site.

A volume of 10µl of each RT-PCR product was run on an agarose gel. As expected all the conditions tested gave a band of ~350bp as shown in Figure 25.
Digestion, dephosphorylation and ligation:
The bands of interest at 350bp were extracted from the agarose gel. The fragments were digested with the BamHI restriction enzyme and purified by agarose gel electrophoresis. As shown on Figure 26, the LC and the HC were visible at the expected size.

In parallel the cloning vector, pBluescript SK (BSK) was also digested with BamHI and dephosphorylated to prevent its recircularization during ligation. The map of BSK is described in the appendix part.

Ligation of the insert fragments to the vector was performed as described above.

Transformation and analyse of the DNA sequence
XL-1 blue bacteria were transformed with each ligation product and bacteria were plated on IPTG-XGal agarose plates. From each condition, 4 colonies were picked and grown for plasmid extraction (§1.3.5) and the plasmid DNAs were sequenced with the upstream T7 sequencing primer. All colonies analysed gave the predicted heavy or light chain N-termini and one colony of each was chosen for further study.

Sequences of the OX-26 variable region kappa light chain (V_L) and the variable region heavy chain (V_H) are shown in the Figure 27 and Figure 29.

**Figure 27: DNA sequence of the variable region heavy chain OX-26**
BamHI sequence in bold, 390 nucleotides

GGATCC CAGGTTCAACTCCAACAGCAGCTGGCTGGGCTGGAGGCTCTGCAAGGCTGTCCTGCAAGGCTTCTGGCTACTCC
TTCCAGCTACTCGGATGGAAGCTGGTAAGCAGAGGCTGGGACTGGTAGATGGCATGATCTATCCTTCTCCGATATGGAGTT
AGGTTAATCACGAAATTCAGCAAGAAGGCAACATGGATGATGGCAACATCATTCCAGACACCACACTACAGCAACTCAACAGCCTAG
GGCGTC

**Figure 28: Amino acid sequence of variable region heavy chain OX-26**
Sequence highlighted are the CDR1, 2 and 3 HC

GSDIQLNQSPSSLSASLGDTILITCHASYNSSTTVLQMNWVKQRPGQLEIGMLPSSDSDLFLRNQKFKDQATLTVDSSTAYMLNS
PTSEDSDAVYCPFRGLDWMGQGTLTVSSAKTTPSVFPLG

**Figure 29: DNA sequence of the variable region kappa light chain OX-26**
BamHI sequence in bold, 367 nucleotides

GGATCC GATATCAGTTGAACAGCTCCTCCAATCAGCTGCTGTGCTGATCCCTGCTGACATCCAGTGCATGCCAGTCAGGC
ATTATGTGGTTAGTTGCTCAGCAGACAGAAGAATGCTGACTAATAGCTGACTAACCTTCAACTTCAACTACAGCTGCAACTCTGC
CCATCAGAGATTAGTGGACAGTCTGGACACAGCTTGCACACACACTGATCCTGAGACAGAAGAATGCTGACTAACCTTCAACTTCA
AACACGGGTCACAGGTCAGGTCAGGGGACCAAGGCTGAAATCAACGGGCTGATGCTGACCAACTCTGATACCTCCATCTCC
CGGATCC

**Figure 30: Amino acid sequence of the variable region kappa light chain OX-26**
Sequence highlighted are the CDR1, 2 and 3 LC

GSDIQLNQSPSSLSASLGDTILITCHASYNSSTTVLQMNWVKQRPGQLEIGMLPSSDSDLFLRNQKFKDQATLTVDSSTAYMLNS
YYQQQSSYFWTFGGGTKLIEKRAADAPTVSIFRIX
1.4.2. Cloning VL OX-26 and VH OX-26 of into pTT5 vectors to express recombinant OX-26

In order to express OX-26 as a recombinant antibody, LC and HC were also cloned into the expression vector, pTT5. New primers were designed carrying the appropriate restriction sites (SalI, BsiWI, BamHI, or NheI at the 5’ and 3’ ends of the V\textsubscript{H} or V\textsubscript{L} OX-26 primer sequences respectively). The LC and HC from the BSK clones were amplified with these new primers as shown in the Table 8.

<table>
<thead>
<tr>
<th>V\textsubscript{L}</th>
<th>V\textsubscript{H}</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA template: plasmid DNA from V\textsubscript{L}</td>
<td>DNA template: plasmid DNA from V\textsubscript{H}</td>
</tr>
<tr>
<td>V\textsubscript{L} top strand primer with SalI site (MS1215)</td>
<td>V\textsubscript{H} top strand primer with BamHI site (MS1217)</td>
</tr>
<tr>
<td>V\textsubscript{L} bottom strand primer with BsiWI site (MS1216)</td>
<td>V\textsubscript{H} bottom strand primer at with NheI site (MS1218)</td>
</tr>
</tbody>
</table>

Table 8: PCR preparation for pTT5 cloning

![Figure 31: 0.8% Agarose gels showing the VL and VH PCR products from OX-26](image)

As shown on Figure 31A, the bands of interest at about 350bp were excised and purified. Digestions were performed by first incubating the VkL PCR fragment with BsiWI in NEB Buffer 3 for 3h at 55°C and then by adding BSA and Sal I, which were incubated O/N at 37°C. The V\textsubscript{H} PCR fragment was incubated O/N at 37°C with the 2 restriction enzymes (BamHI and NheI) and NEB Buffer 2 and BSA. The digestion was checked by gel electrophoresis and the bands of interest were excised and purified as shown in Figure 31B. pTT5 vectors (#18974 for the VkL and #18972 for the V\textsubscript{H} described on Figure 32) were also digested with the same enzyme pairs (data not shown).
Two separate ligations were performed as described in the Table 9. Then XL1-Blue bacteria were transformed with the ligation products and bacteria were plated on LB+Amp agar plate. After O/N incubation at 37°C, 8 colonies from each were picked and grown for DNA minipreps. Plasmid DNA purification was performed and the sequencing of the insert was performed using primer pTT-R and pTT-F. One clone from each set with the correct sequence was selected and grown for large scale preparation of plasmid DNA. The 2 maxipreps #951 and #952 corresponding to pTT5-OX26-VkL and pTT5-OX26-VH were prepared and the sequences were verified by DNA sequencing.
1.4.3. **Cloning of scFv OX-26 into pHal14**

In the scFv, the heavy and light chains of an antibody are combined in a single polypeptide chain separated by a flexible linker. However, the orientation of the heavy and light chains can be critical in order to achieve correct folding to enable reconstitution of the antibody specificity. For this reason, we have combined the OX-26 heavy and light chains in both orientations i.e. \( V_L \)-linker-\( V_H \) or \( V_H \)-linker-\( V_L \). The two variable regions were separated by a 17 amino acid flexible linker (GSGGGSGGASGGGSGGG) and inserted in the pHal14 vector. The composition of the PCR reaction mixes is as follows:

<table>
<thead>
<tr>
<th>DNA Template</th>
<th>Direction ( V_L)-( V_H ) with GS linker</th>
<th>Direction ( V_H)-( V_L ) with GS linker</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer 1</td>
<td>PCR n°1 MS1220</td>
<td>PCR n°2 MS1233</td>
</tr>
<tr>
<td>Primer 2</td>
<td>PCR n°1 MS1240</td>
<td>PCR n°2 MS1241</td>
</tr>
<tr>
<td><strong>PCR product</strong></td>
<td>PCR product 1 ( V_H ) (mp#21)</td>
<td>PCR product 2 ( V_L ) (mp#7)</td>
</tr>
</tbody>
</table>

**Table 10: Strategy of the PCRs**

The primers MS1240, MS1241, MS1242 and MS1243 contain the GS linker. Sequences of the oligonucleotides are given in the Appendix.
Figure 33: Strategy of the PCRs to obtain scFv Ox-26 phagemid

A: Initial BSK vector containing either the VH or the VkL chain; B: Intermediate PCR; C: final PCR product; D: scFv fragment inserted into pHal14 vector after the digestion and the ligation steps.

The PCR n°1, n°2, n°4 and n°5 were performed with 25 cycles and an extension step at 72°C for 30sec and the PCR n°3 and n°6 were performed with 25 cycles and an extension step at 72°C for 45sec. The major bands in Figure 34 A and B shows bands at the expected size.

PCR products of the PCR n°3 and PCR n°6 containing the assembled scFv cassettes and the pHal14 vector were digested with Ncol and NotI restriction enzymes and the bands of interest were purified (Figure 34C) and cloned into the pHal14 phagemid vector.
Figure 34: 0.8% agarose gels to clone scFvs OX-26 into pHal14 vector

PCR products #1 and #5 correspond to the VkL and PCR product #2 and #4 correspond to the V_H. The PCR products #3 and #6 correspond to the scFv V_L- V_H and scFv V_H- V_L with the GS linker respectively. The expected size of the scFv with the GS linker is 786bp.

Fig. A: and Fig. B: analytical gel of the intermediate PCR products Fig. C: Preparative gel of the digested vector (V) and of the final scFvs construction

The ligation products were transformed into DH5α bacteria and colonies for miniprep DNAs were grown in 2YTAG at 30°C. Plasmid DNAs were sequenced and minipreps matching the expected sequence were obtained. The miniprep #11 is OX-26 V_KL-GS-V_H and the miniprep #24 is OX-26 V_H-GS-V_KL.

To conclude, the scFvs of OX-26 in both orientations (V_KL-V_H and V_H-V_KL) have been constructed into pHal14 vector and both versions will be used to produce phage displaying scFv OX-26 at its surface.

2. Production and purification of recombinant proteins

2.1. Material for protein production and purification

The following products have been used: Human Embryonic Kidney 293 cells or HEK293 (Sigma, # 85120602); Nutrient Mixture F-12 (DMEM/F12) (Invitrogen, #21331-020); Fetal Bovine Serum (FBS) (Invitrogen, #16140-071); Penicillin-100µg/ml Streptomycin (Invitrogen # 15070-063); L-Glutamine (Invitrogen, #25030-024 or Gibco, #21331-020); 0.05% Trypsin-0.5mM EDTA 1X (Invitrogen, #25300-054); StemPro® Accutase® Cell Dissociation Reagent
2.2. Culture of HEK293

HEK293 cells were used for protein production following transient transfection, or as a control cell line since kidney cells express many proteins in common with the choroid plexus. They were grown in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (1:1) (DMEM/F12), 10% heat inactivated FBS, 100U/ml Penicillin-100µg/ml Streptomycin, 2mM L-Glutamine at 37°C in 5% CO₂ and passaged twice a week at about 75% confluency by making a 1:10 dilution following detachment for severalmin at 37°C with 0.05% Trypsin - 0.5mM EDTA.

Transient transfection of IgG with the Mirus

To produce recombinant IgGs, plasmids expressing the corresponding heavy and light chains were co-transfected into HEK293 cells using the Mirus TransIT-LT1 transfection reagent. A volume of 250µl of DMEM was mixed with 5µl of Mirus and incubated for 5min at RT. DNAs encoding the light and heavy chains (1.25µg each) were added and incubated for 20min at RT. The mix containing the Mirus, the medium and the DNA was transferred into HEK cell growth medium in a 6-well plate containing about 1E5 cells/well. Cells were incubated for 1 day at 37°C and the IgG was purified from the culture supernatant by chromatography on Protein A beads (§2.5.2).

2.3. Culture of OX-26 hybridoma

The OX-26 rat hybridoma secretes the antibody OX-26 which recognizes both mouse and rat CD71/transferrin receptor (TfR). Cells were grown in suspension culture in RPMI 1640, 2mM glutamine, 10% Fetal Bovine serum at 37°C in the presence of 5% CO₂. Cells were seeded at 5E4 cells/ml and subcultured after 3 days when the cell density reached about 1E6 cells/ml.
2.4. Protein purification

2.4.1. Purification of 6-HIS tagged proteins

Cell culture supernatants were collected and centrifuged at 4000rpm for 10min. Supernatant was recovered and transferred into a new tube. 1ml of the 50% Ni-NTA His-Bind slurry was added to 1ml of 1X Ni-NTA Bind Buffer and mixed. Resin was settled by gravity and the supernatant was removed. A volume of 37ml of the culture supernatant was added to the Ni-NTA His-Bind slurry and incubated at 4°C for 60min on a rotary shaker at 200rpm. The beads were transferred to a Poly-Prep column with the bottom outlet capped. Bottom cap was removed and the flow-through was sampled for analysis. Columns were washed twice with 4ml of 1X Ni-NTA Wash Buffer. Washed fractions were also collected for analysis. Proteins were eluted by adding 4 times 0.5ml of 1X Ni-NTA Elution Buffer in new collecting tube. All the fractions were analysed by SDS-PAGE.

2.4.2. Purification of IgG with protein A

Protein A has a high affinity for the Fc fragment of an antibody and is widely used to purify IgGs. Culture supernatants containing the secreted antibody from OX-26 or transfected HEK293 cells were harvested and centrifuged at 4600rpm for 5min to eliminate the remaining cells and cell debris. Streamline rProtein A resin beads were washed twice with the same volume of 1X PBS, beads were recovered by centrifugation. A 50% slurry of protein A beads was prepared in 2X PBS pH7.4. 1ml of the 50% slurry beads was added to 100 ml of culture medium and incubated overnight at 4°C on a roller to promote the binding between IgG and protein A beads. The next day, beads were transferred into a Poly-Prep column and packed by gravity flow. Beads were washed with 10ml (about 10 bed volumes) of 2X PBS pH7.4 and with 3ml volumes of 100mM NaCl, 25mM Tris pH7.4 and then the IgG was eluted with 3ml of elution buffer (100mM NaCl, 50% ethylene glycol, 10mM Na citrate pH3.5). The eluate was collected and adjusted immediately to pH7 with a solution of Tris 1M pH8. The quantity the IgG in the different fractions was determined by measuring the OD_{280}. Antibodies were filtered and stored at 4°C.
2.5. **Determination of protein concentration**

The concentration of purified proteins was determined by measuring the absorbance at 280nm with the Nanodrop. The same method was used to determine the concentration of total protein in a solution. For antibodies an extinction coefficient of 13.7 was used.

2.6. **Production and purification of hybridoma OX-26**

OX-26 hybridoma cells were cultivated in suspension (§2.3) and the culture supernatant containing the antibody was collected for purification of OX-26 by protein A chromatography (§2.4). All the purification fractions (wash, dead volume, 5 elutions fractions) were analysed on a polyacrylamide gel under reducing conditions to check for the presence and purity of the OX-26 antibody (Figure 35).

![Figure 35: Verification of the purity by SDS-page](image)

The polyacrylamide gel was run under reducing condition and stained with Coomassie Blue. MW: Molecular weight, W: Wash fraction, DV: Dead volume fraction, E1-E2: Elution fraction 1 to 5.

Analysis of the polyacrylamide gel (Figure 35) shows that the fraction Elution 4 was the most highly purified, the gel showing strong enrichment of the two bands at 25kDa and 50kDa, corresponding respectively to the light and heavy chains of the antibody. The other elution fractions had varying amounts of other non-specific contaminants and were discarded. The protein concentration of Elution 4 fraction was 0.56mg/ml as measured by the absorbance at 280nm using the Nanodrop. A larger scale culture of OX-26 (350ml at a final density of 8E5cells/ml) was prepared for antibody production.. The supernatant was filtered and passed over a protein A column and size exclusion chromatography (SEC). The
peak of interest was concentrated to 7.5ml at a concentration of 0.86mg/ml (Figure 36) and used for further studies. Total hybridoma RNA for RT-PCR was extracted from the cell pellet (§1.4).

![Image](echantillon_1.png)

**Figure 36: Measure of the absorbance of the elution fraction of the large scale batch of OX-26 after purification by SEC**

### 2.7. Production and purification of recombinant OX-26

The cloned the LV and HC of OX-26 (§1.4.2) were co-transfected into 50ml suspension cultures of HEK293 cells using 25µg of each plasmids and grown for 3 days. The OX-26 antibody was then purified and as a result, we obtained a concentration of 2.84µg/ml. This batch was then tested by FACS for binding to Z310 cells (Results §1.2).

### 3. Phage display

#### 3.1. Material and preparation of the reagents for phage display

**scFv antibody library:** For the thesis, main of the work has been performed using a HAL7: lambda light chain sublibrary cloned into pHAL14. This library comes from human antibody library containing antibody genes from 44 healthy donors. The heavy and light chains of the variable region were amplified and assembled randomly. The HC and the LC are linked by a peptidic linker. This is a phage library displaying scFv format fragment fused to a His tag, and a c-myc tag, where the Yol linker\(^{114}\) has been replaced in house with a GC rich linker. It
as been calculated that this sublibrary has $>2.8 \times 10^8$ independent clones. And according to colony PCR experiments (data not shown), 89% of the HAL7 has full length inserts. The host bacteria of the library is XL1-Blue MRF’ (supE44) (Stratagene, #200230). Aliquot of 1.15E12 phage rescued with hyperphage rescue were kept at -80°C.

To minimise contamination, all experiments involving bacteriophage were performed in a designated laboratory with dedicated equipment which was regularly decontaminated with 1X Virkon® S from Dupont (VWR, # 115-0021).

**Antibiotics:** ampicillin (Sigma, #A9393) was prepared as 1000 fold concentrated stock (100mg/ml), aliquoted and stored at -20°C. kanamycine B sulfate salt (Sigma, #B5264) was prepared as 1000 fold concentrated stock (50mg/ml), aliquoted and stored at -20°C.

**Glucose 40%**: A solution of 40% glucose was prepared by mixing D (+) glucose and 18.2MΩ.cm water. Solution was warmed up to 50°C to accelerate the dissolution. The solution was filtered with 0.22µm filter and can be kept at RT for one month. This solution is not autoclaved.

**2YT Medium**: The 2X yeast extract and triptone (2YT) medium was prepared by mixing 1.6% of casein yeast peptone, 1% yeast extract, 0.5% of sodium chloride. When required, antibiotic or glucose was added to 2YT medium as follows:

- 2YTAG containing 100µg/ml ampicillin, 2% glucose
- 2YTAK containing 100µg/ml ampicillin, 50ug/ml kanamycin
- 2YTG containing 2% glucose

**2YT agar plate**: The 2 YT agar plates were prepared by adding 1.5% bacto agar to 2YT medium in a Duran bottle. The bottle was autoclaved at 120°C for 20min. The agar was allowed to cool to 35°C mixed with supplements as required and cast into Petri dishes (25ml for a 10cm Petri dish and 250ml for a 25x25cm square plate).

**Minimal agar plate**: Bacto agar powder was mixed to MQ Water to obtain a 1.5% concentration and heated up to boiling point with a microwave oven. After cooling down the solution to about 55°C, the other products were added to obtain a final concentration of 20% M9 minimal salts, 5% glucose, 0.2% MgSO₄, 0.01% of CaCl₂ and 0.1% of thiamine-HCl. 25ml of this solution was cast in a 10cm Petri dish and kept for 30min under the hood. Plate can be kept for one month at 4°C.

**PEG Solution**: A solution of 20% PEG 4000/2.5M NaCl was used to precipitate phage. The reagents were heated together in a microwave oven to give a cloudy solution which
was then cooled on ice with frequent swirling. The solution turns clear and was filtered with at 0.22\(\mu\text{m}\) filter. It can be kept at RT up to one month.

The other following products were used: Hyperphage M13K07ΔpIII (Progen,# PRHYPE); 1 litre baffled flasks (Corning, #CLS431403-25EA); the ApaNovis Phage Select 20Kit (BioCat, #1908-5-A0); heating block (Thermomixer confort, Eppendorf, #5355); Long Amp tac master mix (Bioconcept, #M0287L); MegaX DH10B T1 Electrocompetent cells (Invitrogen, #C6400-03).

The other following equipments were used: Multi-Cartridges System Endosafe (Charles River, #MCSTM); Limulus Amebocyte Lysate Cartridges Sensitivity: 1-0.01 EU/ml (Charles River, #PTS2001), Limulus Amebocyte Lysate Cartridges Sensitivity: 10-0.1 EU/ml Charles River, #PTS201).

3.2. Methods

3.2.1. Construction of phagemids for positive phage controls

Few, if any proteins have been clearly shown to be transported through the brain barriers. Thus, with the aim of putting in place positive control phage for our \textit{in vivo} panning experiments, we have selected a number of potential candidate molecules for display at the phage surface. The selected cDNAs for the peptides, proteins or antibodies were inserted into the phagemid pHAL14 immediately upstream of the phage pIII protein (Figure 37).

![Figure 37: pHAL14 Vector containing the scFv OX-26 (V\text{H}-V\text{L} order)](image-url)
**Proteins**: In the case of the proteins rRAP, rEPO, rLeptin, rEGF and rLIF for which the sequences were known, we have designed oligonucleotide primers to clone cDNAs by RT-PCR (§ 1.2.2) using restriction sites given in the appendix part.

**Peptides**: For the peptides AVP, Angiopep the coding sequences were reconstructed using 2 complementary oligonucleotides and flanked by the restriction sites.

**Antibody**: For the anti-transferrin receptor antibody OX-26, neither the DNA sequence nor the amino acid sequence were available. The strategy was therefore to purchase the hybridoma and purify the OX-26 mAb from the culture supernatant as described in §2.6. The variable heavy (V₉) and variable light (V₈) chains of the OX-26 antibody were then sequenced ‘in-house’ in order to identify the N-terminal amino acid sequence of each chain (Result part §1.2.1). From this information, primers were designed in order to obtain the corresponding cDNAs from the hybridoma RNA by RT-PCR (§1.2.2). The V₉ and V₈ chain cDNAs were then cloned into the pHal14 vector in order to express a scFv version of the original OX-26. Constructions were made in both V₉-V₈ and V₈-V₉ orientations separated by a 17 amino acids modified GS linker (GSGGGSAGASGGSGGG).

Preparation and sequencing of phagemid DNA:
In practice for bacteria infected with phage, the O/N culture was prepared in a Terrific broth medium (TB + 1X phosphate buffer +2% glucose + 100µg/ml ampicilin) and incubated at 30°C instead of 37°C for non infected bacteria (§1.3.5 and §1.3.6).

### 3.2.2. Bacteria culture and long term storage

E. coli TG1 bacteria were maintained on minimal agar plates and prepared fresh for each experiment. F-pillus is expressed in TG1 strain in order to allow the infection of the phage to the g3p. Full genotype of TG1 is shown in the appendix part. A colony was inoculated into 2YTG medium and incubated at 37°C under agitation (250rpm) up to an OD₆₀₀ of 0.5AU. Under these conditions, OD₆₀₀=1AU is equivalent to 8E8cells/ml. After infection by bacteriophage, the incubation temperature was set to 30°C.

For long-term storage, bacteria are frozen at -80°C in 15% glycerol. Stationary phase cultures or bacteria recovered from an agar plate were resuspended in a solution of 2YT + 2% Glucose. Bacteria were centrifuged for 15min at 4000rpm at 4°C and the pellet was resuspended in a solution of 2YT+2% glucose +15% glycerol (1 volume of the pellet). The OD₆₀₀ should be around 100AU (equivalent to 10E11bacteria/ml). Then bacteria were
aliquoted and snap frozen on dry ice. Bacteria were recovered from a frozen tube by scraping the frozen medium with a sterile toothpick and streaking it on an appropriate agar plate. For a 96 well suspension cultures, 50µl of 50% glycerol in TBAG was added to the 100µl of overnight culture to give a final concentration 16% glycerol stock.

3.2.3. Rescue of the phage
This procedure enables phage amplification. Helperphage M13KO7 promotes the production of monovalent phage in which most Gp3 proteins are derived from the WT phage, whereas hyperphage M13K07ΔpIII (which lacks a p3 gene) allows the production of multivalent phage in which multiple copies of the protein of interest are displayed on the phage surface.

With helper phage
E.coli TG1 infected with phagemid were grown in 2TYAG at 30°C for 1.5-2.5h in baffled flasks until OD600 reaches 0.5AU. The helper phage M13KO7 was added to give a 2:1 ratio of phage to cells in the culture. The flask was incubated for 1h at 37°C with occasional gentle swirling to allow infection. The bacteria were centrifuged at 4000rpm for 15min and the supernatant was removed. The pellet was resuspended in 2YTAK (with ampicillin and kanamycin but without glucose) and grown at 30°C O/N with vigorous shaking. Phage were released into the medium.

With hyper phage
200µl of E. coli TG1 infected with the phage (usually from glycerol stocks) was inoculated into 100ml of prewarmed 2YTAG at OD600=0.1AU and grown at 30°C under agitation (250rpm) for about 2h until OD600 ~0.4-0.5AU corresponding to about 4E08 bacteria/ml. 250µl of Hyperphage M13K07ΔpIII (at 1.0E12pfu/ml) were added to 25ml of the bacterial culture (1.0E10 bacteria) and incubated for 30min at 37°C without shaking and then for a further 30min with shaking at 110rpm. The culture was then centrifuged at 4000rpm for 10min and the supernatant was carefully discarded. The bacterial pellet was resuspended in 5ml of 2YTAK and transferred into 400ml 2YTAK medium in 1 litre baffled flasks. Flask were incubated O/N at 30°C under gentle agitation (170rpm).
3.2.4. **Phage concentration**

**With the PEG method**

Medium from an O/N phage culture (§3.2.5) was centrifuged at 4000rpm for 20min at 4°C to pellet the bacteria and the supernatants containing phage in solution were carefully transferred to a new tube. A volume of PEG 4000/NaCl equivalent to 10% of the supernatant was added and mixed gently by hand. The tube was incubated on ice for 30min to precipitate the phage then centrifuged at 4000rpm for 20min at 4°C (swing out rotor). The supernatant was discarded and the pellet was resuspended in PBS. An equal volume of PEG 4000/NaCl was added and mixed by gently inverting the tube. The solution turns milky white, indicating a high yield of precipitated phage. The tube was kept on ice for 10min and then centrifuged at 4000rpm for 20min at 4°C. The supernatant was gently discarded and the phage pellet was finally resuspended in PBS.

**With the kit from Biocat**

M13 bacteriophage were also purified with the ApaNovis Phage Select 20 Kit. This method is quicker (requires about 1h) compared to the PEG precipitation method but more expensive. The principle is based on the binding of the phage to a membrane (fixed on a spin column) which can be washed and from which the phage can be eluted (pH dependant). The eluate is then diluted in PBS and concentrated on a 100kDa cut-off spin column to standardize the buffer. The phage obtained are free from PEG and can be used directly for titration and further rounds of panning.

3.2.5. **Lipopolysacharide elimination**

Lipopolysaccharides (LPS) are endotoxins released from Gram-negative bacteria and are strong inducers of the innate immune response in animals leading to rapid secretion of pro-inflammatory cytokines. Phage preparations derived from E. coli are massively contaminated with LPS, typically containing greater than 10E6EU/ml whereas for in vivo work, less than 50EU/kg is recommended. In order to reduce the LPS titre for our phage preparations, the following protocol was adapted from Aida et al.\textsuperscript{115, itself adapted from Karplus et al.\textsuperscript{116}. It is based on the use of a detergent, Triton-X114 which has a conveniently low cloud point of 23°C.

The phage preparation was diluted in PBS to 1.0 ml in a 1.5ml microtube. 20µl of 5% Triton X-114 was added to reach a final concentration of 0.1%. The tube was chilled on ice to give
a homogeneous single phase solution and transferred to a roller at 4°C for 30min to solubilize endotoxins. The tube was then warmed at 30°C for 5min in a heating block until the solution turns cloudy (phase separation), then centrifuged at 10000rpm for 3min at RT. The supernatant was withdrawn into a fresh tube and the small oily detergent layer was left undisturbed. A further 20µl of Triton X-114 was added and the procedure was repeated a minimum of 5 times.

LPS titre determination was performed with Endosafe®-PTS: (Charles River, Endosafe) following the protocol from the supplier. The sensitivity of detection depends on the cartridge used and the system can detect within the range 0.01-10EU/ml range.

3.2.6. Phage Titration
To determine the number of phage particles in a solution, the ability for a phage to infect the bacteria was used. Since only one phage can infect bacteria via its F-pilius, the number of viable phage particle able to infect bacteria can be determined as the number of colony forming units (cfu).

Fresh E. coli TG1 from a minimal plate is grown in 2YTG media up to OD\textsubscript{600} of 0.5AU. Serial dilutions of phage in the range of 1E2 to 1E15 were made in 2YTG medium and mixed at a 1:1 ratio (150µl+150µl) with log-phase bacteria. The samples were incubated at 37°C for 1h with gentle swirling every 20min to allow the infection. Bacteria were then centrifuged and resuspended into 1ml of 2YTG media. 1:10 and 9:10 of the solution were spread onto a 2YTAG plate and incubated O/N at 30°C. The next day, colonies were counted on each plate and the dilution factor was applied to obtain the final concentration of phage expressed as cfu.

3.2.7. Recovery of internalized phage from a cell
Recovery of internalized phage was performed according to the Burg protocol\textsuperscript{117}. Briefly, cells were washed with glycine buffer (200mM glycine pH2.2) to remove bound phage. Cells were resuspended in 500µl of lysis buffer containing 1% Triton X-100 diluted in PBS. Cells were triturated with the pipette and keep on ice for 1h. Non solubilized material was pelleted at 15 000rpm for 2min and the supernatant containing the phage was recovered for bacterial infection. Plasmid DNA was extracted using the standard procedure described above.
3.2.8. **Bacterial infection with phage**

Bacteria (E. coli TG1) were grown at 37°C to OD$_{600}$ of 0.5AU as described in the §3.2.2. Phage were added to the bacteria and incubated for 1h without agitation at 30°C with gentle swirling after 20 and 40min. Then the infected bacteria were pelleted at 4000rpm for 20min at 4°C. The supernatant was discarded and the pellet was resuspended into 2YTG. Bacteria were then seeded on agar plate containing the antibiotic for selection (in our case ampicillin), and incubated O/N at 30°C. Each colony represents infection with a single phage particle. The optimal multiplicity of infection (moi) is approximately 10 for a M13 bacteriophage.

3.2.9. **Reformatting scFv into pTT vector**

The aim of this procedure is to convert the $V_L$ and $V_H$ sequences in the scFv insert of an interesting phage candidate into a reformatted IgG. To do this, the variable light and heavy chain inserts are cloned into the respective pTT vectors containing the light or heavy chain framework elements from human IgG. Corresponding pairs of plasmids are then co-expressed in HEK293 cells to enable the production of the IgG. In our library, only lambda light chains are present.

**Figure 38: pTT5 vectors with a $V_H$ and $V_L$ chains from OX-26**

**Fig. A**: pTT5 vector with a variable heavy chain from OX-26; **Fig. B**: pTT5 vector with a variable kappa light chain from OX-26

PCR: The first step was to design the forward and reverse primer pairs for the $V_L\lambda$ chain with FseI and BssHII restriction site extensions, and the $V_H$ chain with NcoI and SalI restriction site extensions. Primer sequences are listed in the appendix. The following reactions were set up for each clone according to Table 11.
For $V_L$ $\lambda$

<table>
<thead>
<tr>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>BssHII For Pool</td>
<td>FseI Rev pool</td>
</tr>
</tbody>
</table>

For $V_H$

<table>
<thead>
<tr>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>UnilacFOR pool</td>
<td>HJSaII_REV pool</td>
</tr>
</tbody>
</table>

**Table 11: PCR Mix for the amplification of the variable lambda region and the heavy chain of OX-26**

Amplification of the variable lambda region and the heavy chain was performed by PCR following the following program (Table 12).

<table>
<thead>
<tr>
<th>Temp</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>94°C</td>
<td>Ever</td>
<td>Insert sample</td>
</tr>
<tr>
<td>94°C</td>
<td>30sec</td>
<td></td>
</tr>
<tr>
<td>94°C</td>
<td>20sec</td>
<td>27 cycles</td>
</tr>
<tr>
<td>65°C</td>
<td>30sec</td>
<td></td>
</tr>
<tr>
<td>65°C</td>
<td>1min 20sec</td>
<td></td>
</tr>
<tr>
<td>65°C</td>
<td>10min</td>
<td></td>
</tr>
<tr>
<td>4°C</td>
<td>Ever</td>
<td></td>
</tr>
</tbody>
</table>

**Table 12: PCR Program for the amplification of the variable lambda region and the heavy chain of OX-26**

Each PCR products were checked by agarose gel electrophoresis, cut and cleaned up (§1.2.5). Digestion of the PCR product and of the new receiving vector were performed (§1.3.1.) according to the Table 13.
The digestion took place for 3h at 37°C. The digested PCR reaction was cleaned (§1.2.5) and the digested vector was run on a 0.8% agarose gel (§1.2.4), the band corresponding to the digested vector was cut off and cleaned up (§1.2.5). Concentration of the cleaned digested vector and insert were determined using the Nanodrop.

Ligation: Ligation was performed according to the Table 14 and incubated for 2h at RT (§1.3.3).

**Table 14: Calculation of the amount of insert for the ligation**

<table>
<thead>
<tr>
<th>V&lt;sub&gt;L&lt;/sub&gt;</th>
<th>V&lt;sub&gt;H&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.4kbp</td>
<td>0.5kbp</td>
</tr>
<tr>
<td>4.726kbp</td>
<td>5.414kbp</td>
</tr>
<tr>
<td>80ng</td>
<td>80ng</td>
</tr>
<tr>
<td>20.3ng</td>
<td>22.2ng</td>
</tr>
</tbody>
</table>

Transformation:

50µl of MegaX DH10B T1 Electrocompetent cells (full genotype in appendix part) were transformed by electroporation (§1.3.4) with 1µl of ligation product. A volume of 1ml of SOC was added to the transformed cells and 100µl were plated on LB plates with ampicillin (100µg/ml). Plates were incubated O/N at 37°C. Single colonies were picked and grown in 1.5ml TB (phosphate) medium+ Amp 100µg/ml O/N 37°C in deep-well blocks. Miniprep
were performed (§1.3.5) to extract the circular DNA and sequencing (§1.3.6) was performed to check the DNA sequence.

### 3.3. Production of scFvs OX-26 phage

scFv OX-26 pHal14 phagemids described above were used to transform cultures of E.coli TG1 by electroporation (§1.3.4) and the corresponding phage were rescued with wild type helper phage as describe in §3.2.3. Then medium containing phage was purified with the Biocat kit (§3.2.4) and the phage yield was titered. We obtained preparations of scFv OX-26 $V_H$-$V_L$ phage with a titer of $2.1 \times 10^{14}$ cfu/ml and scFv OX-26 $V_H$-$V_L$ phage with a titer of $1.7 \times 10^{14}$ cfu/ml. Both phage solutions were used for binding experiments (Result part §1.2.4).

### 4. In vivo model

#### 4.1. Material for in vivo model

The following products were used: isoflurane (Baxter, #T033B72-1A); Rompun® 2 (xylazine) and 75mg/kg of Ketavet® 10 diluted into 0.9% NaCl (Sigma, #S8776). The following equipment was used: 5mm lancet (Goldenrod, #GR-5); ADAM automated mammalian cell counter (Digital Bio, #ADAM-MC); 31G insulin syringe (BD, # 324912); all surgical material (25G needle, 5ml syringe, was cleaned and sterilized before use or a single use). All animals used were Sprague Dawley rats supplied by Charles River Laboratory or Janvier Laboratory.

#### 4.2. In vivo methods

##### 4.2.1. Animal welfare and origin of the animals

All the animal experiments were performed according to the Swiss ordinance on Animal Experimentation as well as the animal protection law. The experimental protocols were approved by the Swiss veterinarian office (Office Cantonal Vétérinaire (OVC) from Geneva, Health general direction).
For pharmacokinetic studies, histology and organ sampling, 200-300g male rats were used under the Swiss authorization 3108/1-2R+C or 1040/3689/2. For preparing primary cultures of the CP, pregnant female at E14 stage (§5.1) were ordered under the authorization 1040/3652/0. Canulated rats with long-term canulation of the cisterna magna were used for CSF sampling under the special authorization 1040/3746/1.

All the protocols where also approved by the Merck Serono Ethical Committee. The animal room was accredited by the AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care International) and the light, food, ventilation, temperature and stabulation were provided in accordance with the “Guide for the Care and use of laboratory animals” from the National Research Council (9th edition). As the person in charge of the experiments, I was trained and accredited by the Swiss authority to perform animal experimentation (module 1).

### 4.2.2. Anaesthesia

Depending on the type of surgery to be performed different techniques for anaesthesia were used.

For intravenous injection in the tail vein, only a light anaesthesia with 3% isoflurane inhalation for 5min was used.

For perfusion and immunohisto-chemistry, a deep anaesthesia was applied. A lethal dose of pentobarbital was injected intraperitonealy at 100mg/kg.

For a normal CSF sampling in the cisterna magna, a deep anaesthesia was performed by combining a first isofluorane inhalation followed by injection of a 10mg/kg of Rompun® (xylazine) and 75mg/kg of Ketavet® 10 diluted into 0.9% NaCl solution. A dose of 1.5 ml/kg of this solution was injected intramuscularly in both thighs.

### 4.2.3. Product administration into the peripheral circulation

Administration of the different products (protein, antibody, phage, dye) has been carried out intravenously in the tail vein under isofluorane anaesthesia (Figure 39). The tail was warmed for several minutes in a 37°C water bath to vasodilate the vein. Alternatively, the entire animal could be heated in a warm cabinet for 10min at 39°C. 100-500µl of product was administrated per adult rat.
4.2.4. **Blood sampling**

The total blood volume for an adult rat is about 60ml/kg. When a small volume of blood was needed, a lateral saphen vein puncture was performed with a 5mm lancet (Figure 40A). For this sampling, thighs of the rat were shaved and the rat was placed into a 39°C cabinet for 5min to increase vasodilatation. This technique is quick and has the advantage that several samplings can be performed from the same site. In this study, this technique was used for sampling up to 24h; if a longer time point was required a surgical canulation was put in place.

When a larger volume of blood was needed, lethal deep anaesthesia was followed by intracardiac sampling using a 25G needle and a 5ml syringe (Figure 40C and D). Up to 8ml of blood could be sampled with this technique.

**Figure 40: Sampling of venous blood in the saphen vein or by intracardiac puncture**

*Fig.A: Sampling at the saphen vein with (Picture extracted from http://www.medipoint.com/html/for_use_on_rats.html). Fig. B and Fig C: Intracardiac sampling with 5ml syringe and 20G needle (picture extracted from http://web.jhu.edu/animalcare/procedures/rat.html)*

4.2.5. **CSF Sampling technique**

In rat, CSF is routinely obtained by aspiration from the cisterna magna (Figure 41).
This puncture has to be performed under deep anaesthesia as described in the §4.2.2 and is lethal. To access the cisterna magna (Figure 41) with a needle, an optimal angle between the head and the back needs to be maintained and no dissection or shaving is performed. The angle is usually formed with the hand or can be done with a modified stereotaxic instrument (Figure 42).

The development of alternative method for CSF sampling is described in detail in the results section §2.2.3.

4.2.6. Dissection
All dissection were performed under anaesthesia (§4.2.2). Insensitivity to pain was tested just before the dissection. Dissection was performed in a sterile environment using sterile instruments. Rats were fixed with adhesive tape to expose the organs of interest. Skin and hair were sprayed with 70% ethanol. Perfusion will be described in the §7.4.1 concerning histological procedures.
5. In vitro transport models

5.1. Material

The following products were used: Pronase (Sigma, #P5147); PBS (Gibco, #10010-023); 250µg/ml trypsin (Gibco, #27250-018); 1.25µg/ml DNase I (Sigma, #AMPD1); Ham’s F12 (Lonza #CE12615F); DMEM (Gibco #41965); Fetal bovine serum (PAA #A15-503); glutamine (Gibco #25030); gentamycine (Gibco #15750-037); hypoxanthine (Sigma #H936); hydrocortisone (Sigma #H0888); FGF (Biosource #PMG0034); EGF (Biosource #PMG8045); insulin, transferin, selenium (Gibco #41400-045); Dynabeads M-280 streptavidin (Invitrogen, #112.05D); Dynabeads oligo(dT)25 (Invitrogen, #610.02); Dynamag-2 magnetic particale separator (Invitrogen, #123.21D); 200ng/ml HRP-biotin (Pierce, #0029139); TMB HRP substrate (Uptima, #UP664780); Tween® 20 (Sigma, #27434-8); Bovine serum albumin (BSA) (Sigma, #A9418); Avidin (Tetrameric)-Sepharose CL-4B (Affiland, #BE AVI-T-4S); Advance DMEM (Gibco, #12491-015); penicillin-streptomycin (Gibco, #15070-063); 50mg/ml gentamicin solution (Gibco, #15750-078); 100µg/ml Recombinant mouse epidermal growth factor (EGF) (Biosource, # PMG8045); trypsin solution (Gibco-Invitrogen, #25300-054); Matrigel (BD Bioscience, #356234).

The following equipment was used: Transwell® Polyester (PET) with a 0.4µm pore size,12mm diameter and a 1.12cm² surface (Costar, #3460); or with a 6.5mm diameter for a 0.33 cm² surface (Costar, #3470); cell strainer with 100µm pore (BD Falcon, #352360). Petri dishes, flasks and pipettes and tubes were supplied by BD Falcon. Centrifuge, incubator and water bath were dedicated used solely for mammalian cells. All cell culture was performed in a dedicated laboratory under a laminar flow hood and all the instruments were sterile. All the media and supplements were warmed at 37°C in the water bath before use. Cells number was determined with an ADAM automated mammalian cell counter (Digital Bio, #ADAM-MC).

Pregnant Sprague Dawlay rats were obtained from R.Janvier SAS and were received at E14. About 12 pups were used from the age of P7 to P10 (post natal day 7 to 10).
Z310 cells line has been established by Dr Zheng in 2002 by immortalization of a rat choroidal epithelial cell. Z310 were kindly obtained from Dr Zheng (Purdue University).

**Preparation of solutions**

A solution of 1mg/ml pronase diluted in sterile PBS was prepared and aliquoted and kept frozen at -20°C.

A solution of 250µg/ml trypsin and 1.2 µg/ml DNase I diluted in sterile PBS was prepared and aliquoted and kept frozen at -20°C.

Culture medium containing 50% Ham’s F12 (Lonza, #CE12615F), 50% DMEM (Gibco, #41965), 10% Fetal Calf Serum (FCS) (PAA, #A15-503), 2mM Glutamine (Gibco, #25030), 50µg/ml Gentamycine (gibco, #15750-037), 500µM hypoxanthine (Sigma, #H936), 2µg/ml hydrocortisone. (Sigma, #H0888), 10ng/ml FGF (Biosource, #PMG0034), 5ng/ml EGF (Biosource, #PMG8045), 1x ITS (insulin, transferin, selenium), (Gibco, #41400-045) was prepared and filtered at 0.22µm and kept at 2-8°C.

ELISA wash buffer (EWB) containing 25mM Tris pH 7.5, 150mM NaCl, 0.05% Tween® 20 (Sigma, #27,434-8), 0.1% BSA (Sigma, #A9418) was prepared and stored for up to one month at 4°C.

The Z310 culture medium was composed by DMEM, 10% FBS, 10% penicillin-streptomycin, 0.4% gentamicin solution and 10ng/ml EGF.

### 5.2. *In vitro* methods

#### 5.2.1. Primary culture from choroid plexus epithelial cells

Historically, primary choroid plexus epithelial (CPe) cells were first obtained from bovine brain as it was easier to extract and to isolate CP organ from larger animals. This method combined mechanical and enzymatic treatments to obtain isolated CPe cells. In 1989 Tsutsumi et al. adapted this existing protocol for the rat choroid plexus. A similar protocol has been used for porcine CP and for human CP. In 1999, Strazielle et al. further modified the protocol, the main changes being the use of 1-day old pups instead of 20-day old pups, and minor changes in the digestion conditions. Using this protocol, we initially attempted to passage cells to increase cell biomass however a large proportion of the
cells failed to re-adhere to the culture surface after detachment thus all subsequent experiments were performed with primary, non-passaged cells.

The dissociation of the cells could be performed by enzymatic treatment (pronase, trypsin, DNase I, collagenase, dispase) or mechanical trituration. The culture support could also be different in the material and a coating (collagen, laminin, Matrigel®,...) could be added to mimic the basal lamina. Optimization of the conditions for primary cultures is described in the results section.

Dissection of the choroids plexus from the brain

Pups aged from P7 to P10 were decapitated with surgical scissors and the heads were kept in 10cm Petri dishes. Heads were sprayed with 70% ethanol. With small scissors and small forceps, skulls were opened and the brains removed and collected in PBS. The brain was cut with a scalpel in sagital manner to expose the two hemispheres. The CP from the fourth ventricle (4V) was removed with a thin forceps and stored at 4°C in a tube containing complete growth medium. The cortex was opened to expose the choroid plexus surrounding the lateral ventricles which was also extracted and stored in a separate tube.

Figure 43: Dissection of the choroid plexus

**Fig. A**: Instrument use for choroid plexus extraction. **Fig. B**: Skin incision to expose the skull. **Fig. C**: Exposure of the brain before its transfer in a PBS Petri dish. **Fig. D**: Sagital section of the brain with a scalpel to expose the lateral ventricle. **Fig. E**: Dissection of the choroid plexus was performed under binocular microscope. **Fig. F**: Dissected choroid plexus from the lateral ventricle.
Extraction of the choroid plexus epithelial cells

The CP tissue was transferred into a 1.5ml microtube and left for 1min on the bench to enable the organs to settle to the bottom of the tube. The medium (supernatant fraction) was gently removed and replaced with 1ml of PBS. This washing step was repeated twice to remove any single cells in suspension (mainly blood cells).

After the last rinse, the PBS was removed and replaced with 1ml of pronase solution. The mixture was then rotated for 25min at 37°C to allow a first disruption of the tissue. After the incubation, CP was transferred to a cell 100µm nylon mesh (Figure 44) in a 6 wells plate and rinsed twice with 5ml of PBS. The liquid which passed through was discarded.

The 100µm nylon mesh containing the choroid plexus was then transferred into a clean well and 6ml of trypsin/DNase I was added to cover the tissue. The mixture was incubated for 3min at RT with smooth orbital agitation and the 100µm nylon mesh was rinsed with 2ml of PBS. This step separates the too large cluster of CP cells from the optimal size cluster. 1ml FBS was added to neutralize the action of the trypsin.

The 9ml were collected into a 50ml tube. This digestion steps with trypsin/Dnase I was repeated three more time.

Figure 44: 100µm nylon mesh inserted into a 6-well plate

The tube containing cells (around 36ml) was centrifuged 8min at 400rpm. The supernatant which contained mainly single cells and fragments of cells was discarded and the pellet was resuspended in 1ml of complete medium.

The purity of the CPe will be achieved either by selective attachment (fibroblast adhere faster that CPe) or by selective culture (D-Valine for L-valine substitution inhibit fibroblast proliferation (Gilbert et Migeon 1975) or a combination of both. Based on the results from 4,
we have decided to use only the selective attachment. In practice, cells were seeded in 1 well of a 24 well plate for 2h at 37°C, 5% CO₂ to enable preferential adhesion of the fibroblasts. This pre-attachment step was used to deplete fibroblasts from the CP cells.

During this time, the transwells® were coated with Matrigel® which is a matrix containing high level of laminin with also nidogen, entactin and collagen IV: pure Matrigel was thawed on ice and diluted at 1:40 in cold Ham's F12/DMEM to obtain a concentration of 0.25mg/ml. 200µl (for the 12mm diameter transwell®) or 100µl (for the 6.5mm diameter transwell®) of diluted Matrigel® were transferred into the upper chamber of the transwell® and incubated for 1h at RT. Excess of Matrigel® was removed by aspiration just before seeding with CP cells.

After the fibroblast pre-attachment step, the supernatant was transferred into a new well and the CP cells were counted using a method based on the DNA measurement since most CPe cells were present in clusters. Cells were seeded in a transwell® at a density of 5E5 cells/cm² and incubated at 37°C in the presence of 5% CO₂.

The medium was changed every 1-2 days depending on the confluence. The TEER was monitored with the CellZscope (§5.2.4) and the level of the liquid was checked every day. The CPe cells coming from the 4V or the LV were handled separately.

5.2.2. Culture of choroidal epithelial Z310 cells
Z310 cells were seeded at a density of 25.10E3 cells/ml and grown in normal Petri dishes in a 5% CO₂ incubator at 37°C without surface coating. Cells were split twice per week at about 80% confluency by washing with PBS, releasing cells by adding 0.05% trypsin and subcloning at a dilution of 1:16 to 1:20.

5.2.3. Dual chamber system and transport assay
A dual chamber system makes it possible to separate physically two fluidic compartments. This method is often used to mimic a physiological barrier like the gut or brain barriers. We used 0.4µm pores to prevent cell migration from one compartment to the other. We used polyester membrane in order to be able to visualize the density of the cells which is not possible with polycarbonate membrane. For experiments with primary cells, we have used
small transwells of 0.33cm² (6.5mm diameter) and for experiments with cell lines, we have used larger transwells of 1.12cm² (12mm diameter).

![Figure 45: Dual chamber system](image)
From Costar manufacturer guideline

For transport experiments, the choice of the control depends on the kind of molecule under study. In our case, we want to study the transport of large molecules and more specifically the transport of antibody-derived molecules.

5.2.4. Measure of the TEER

The transepithelial electrical resistance (TEER) represents the resistance of the epithelial barrier and was measured continuously under physiological conditions using the CellZscope® (Supplier Nanoanalytics). It is expressed in ohm.cm² and an increased in TEER is correlated with the formation of TJ:s. The measure of the capacitance in microfarad (µF) is also possible and the reduced capacitance indicates the formation of a cell monolayer. This apparatus allows us to monitor simultaneously up to 24 parallel cultures and for our experiments, one measure was performed every 15min. The machine was used according to manufacturer protocol.

![Figure 46: CellZscope equipment](image)
Picture extracted from Nanoanalytic supplier
6. Biochemical methods

6.1. Material

The following products were used: Alexa Fluor® 488 protein labeling kit (Invitrogen, #A10235); Triton X-100 (Sigma, #T8787); HEPES (Sigma, #90909C); Sucrose (Merck Millipore, #1076871000); DL-Dithiothreitol (DTT) (Sigma, #43815); Complete Protease Inhibitor Cocktail Tablets (Roche, #11836153001); ethylenediaminetetraacetic acid (EDTA) (sigma, #E6758); tris(hydroxymethyl)aminomethane (Tris) (Sigma, #154563). For the gel electrophoresis and immunobloting, all the reagents used were from Invitrogen (NuPage 10% Bis–Tris 1mm gels (Invitrogen, #NP0302BOX), antioxidant, nitrocellulose membrane, reducing agent, SDS sample buffer, SDS running buffer and SDS transfer buffer, ponceau red, Coomassie blue); 25% trifluoracetic Acid (TFA) (Applied Biosystems, #400028); Chemoluminescence ECL detection kit (GE Healthcare, #RPN2209); Prestained Protein Molecular broad low range (20,7–103 kDa) (Bio-Rad, #161-0305) and high range (48–204 kDa) (Bio-Rad, #161-0309).

The following equipment was used: cell disruption vessels (Parr Instrument, #4639) and its Nitrogen cylinder; ultracentrifuge (Beckman TL100) and the associated tubes TL100 and TLA100; Precise protein sequencer (Applied Biosystem, #ABI494).

6.2. Extraction of membrane proteins

Two types of method can be used to extract membranes proteins: a detergent-based methods and a detergent-free method using nitrogen cavitation.

Extraction using detergent

Medium from adherent HEK293 cells was removed and the cells were washed once with 1X PBS. PBS was removed and cells were harvested with a cell scraper. Cells were centrifuged for 5min at 1500rpm and 1ml of fresh lysis buffer (1% Triton X-100; 10mM HEPES; 270mM Sucrose; 1mM DTT; 1X protease inhibitor) was added to the pellet. Cells were pipetted up and down gently several times with a 1ml pipette tip and the lysed cells were centrifuged for 10min at 5500rpm. The pellet is termed the post nuclear supernatant (PNS) and was washed with 1ml of 1X PBS and centrifuged for 5min at 1500rpm. The PNS
was transferred to a TL100 tube and ultracentrifuged for 1h at 50000rpm. The high-speed supernatant contains the cytosolic proteins while the pellet represents the crude cytoplasmic membrane fraction. For electrophoretic analysis, the membranes are solubilized in 2X SDS-Page loading buffer.

By nitrogen cavitation

Harvesting Cells: Adherent cells were harvested using a cell scraper and transferred into a 50ml tube. Cells were pelleted by centrifugation for 10min at 1000rpm and the supernatant was removed. The cell pellet was resuspended in 20ml of Cell Cavitation buffer (250mM Sucrose, 50mM Tris pH 7.5, 1mM EDTA, 1mM DTT, 1X Protease Inhibitor cocktail freshly prepared), again pelleted by centrifugation for 10min at 1000rpm and resuspended in 10ml of Cell Cavitation buffer.

Nitrogen cavitation: Cells were placed in the chamber of the nitrogen bomb. Valves were closed and the pressure control valve was gently opened until a pressure of 800psi was obtained. The chamber was left on ice for 30min to equilibrate the pressure inside and outside the cells, then the pressure was released and the lysate was expelled into a tube.
placed at the base of the collection valve. The lysate was centrifuged for 5 min at 500g to pellet the unbroken cells and nuclei. The supernatant was carefully removed.

Ultracentrifugation: The Beckman centrifuge, TL-100 rotor and TL100 tubes were precooled to 4°C and 1 ml aliquots of the lysates were placed into polycarbonate TL-100 tubes and membranes were pelleted by centrifugation for 60 min at 51 000 rpm (~100 000 x g) at 4°C. Each pellet was resuspended in 100 µl of buffer and pooled.

6.3. Gel electrophoresis and immunoblotting

The Novex® NuPAGE® SDS-PAGE Gel System from Invitrogen has been used for SDS-PAGE and western-blot and the protocol from this supplier was followed. Electrophoresis was routinely performed in 4-12% polyacrylamide for 35 min at 200 volts using the running buffer supplied by the manufacturer. Proteins were transferred electrophoretically to PVDF membranes at 100 V for 30 min in transfer buffer and membranes were removed and air dried. Non-specific binding was blocked by incubation in 0.1% Tween 20 5% milk in TBS buffer for 1 h at RT. Primary antibody was incubated O/N at 4°C or 2 h at RT. The secondary antibody linked to HRP was incubated 1 h at RT and detection was performed using the ECL reagent (GE Healthcare) in a dark room using film (GE Healthcare).

6.4. N-terminal Sequencing

For antibodies, the two chains were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The bands corresponding to the HC and the LC were cut out and solubilized. N-terminal amino acid analysis has been performed using the Procise protein sequencer performed by our in house analytical service using Edman Sequencing technique. 200 pmol of proteins were diluted into 100 µl of 0.1% TFA. 10 µl of methanol were added to a Prosorb Insert to wash the membrane and 100 µl of the protein diluted in 0.1%. The membrane of the Prosorb Insert was dried, cut and transferred in a clean cartridge of the sequencer. The cartridge was then put in the sequencer. Through the software Procise, select the number of cycles a run the experiment. Data were analysed with SequencePro software after checking the calibration.
7. Immunological methods

7.1. Material

The following products were used: PBS pH7.4 (Gibco, #10010-015); Tween® 20 (Sigma, #27,434-8); Bovine serum albumin (BSA) (Sigma, #A9418); TMB ELISA peroxidase substrate (Uptima # UP664781); sulfuric acid (H₂SO₄) (Sigma, #339741); Hoechst dye diluted at 1:2000 (Invitrogen, #H1399); Vectastain Elite ABC Kit (Universal (Vector Laboratorie, #PK-6200); Eukitt mounting medium (sigma, #03989); paraformaldehyde (PFA) (Sigma, #158127); sodium hydroxide (NaOH) (Sigma, #S8045); sodium phosphate dibasic (Na₂HPO₄) (Sigma, #S3264); sodium phosphate dibasic dihydrate (NaH₂PO₄.2H₂O) (Sigma, #71643); sodium azide (Sigma, #S2002).

The following primary antibody were used: rabbit anti-ZO-1 antibody (Zymed, #61-7300), rabbit anti-Claudin-1 antibody (Zymed, #71-7/00), rabbit anti-transthyretin antibody (Santa Cruz, #sc-13098); rabbit anti-LRP2 H-245 (Santa Cruz, #sc-25470); goat anti-LRP2 P-20 (Santa Cruz, #sc-16478).

The following secondary antibodies were used: Goat anti-human kappa Light chain HRP conjugated (Millipore, #AP506P); Goat anti-human IgG, Fcγ fragment specific : (Jackson Immunoresearch # 109-005-098); Goat Anti-Rabbit AF488 linked (Invitrogen, # A-11070).

The following equipments were used:MaxiSorb 96 well plates (Nunc, #475094); inverted fluorescent microscope (Leica); BD FACSCalibur flow cytometer (BD Biosciences) and FlowJo software (FlowJo); Vibratome (Leica Microsystems, Leica VT1200S); Cryostat (Microm, #HM560); Blades for cryostat (Richard-Allan Scientific, #152215); Superfrost glass slides (Thermofisher, #12-550-15); tissue mounting fluid for cryostat (OCT embedding compound (Tissue Tek, #4583); cyanoacrylate glue for vibratome (Henkel, #sicomet 50); Pain brushes; Gillette super silver blades (Gilette, #GIN446151, SPA13270224).

Preparation of 2% PFA in 0.15M phosphate buffer pH7.4 for perfusion: 40g PFA were dissolved in 625ml dH₂O and warmed to 60°C while stirring continuously until entirely dissolved. If the solution is not clear, 10M NaOH was added dropwise while stirring continuously until entirely cleared. Then 375ml of 0.4M Na-phosphate buffer was added (Sodium phosphate stock solutions contain for one liter 71.2g of Na₂HPO₄ and 62.4g of
NaH₂PO₄.2H₂O). The solution was filtered and cooled to RT, the pH was adjusted to 7.4, and the solution left on ice until use.

### 7.2. FACS

FACS staining buffer (FB) was as follows: 1% BSA + 0.1% NaAzide diluted in PBS. For adherent cells, cells were detached preferentially with accutase, centrifuged for 3min at 1000g and washed once with PBS. After counting of the cells, 2E5 cells were transferred into a V-bottom 96 wells plate. The plate was then centrifuged for 3min at 1000g and the supernatant was discarded by flipping the plate once. Cells were resuspended with 100µl of primary antibodies at 5µg/ml and incubated for 1h on ice. Cells were washed twice with 200µl of FACS buffer and resuspended with 100µl of secondary antibodies linked to a fluorophore at 1:100 dilution and incubated for 1h on ice. Cells were washed twice with 200µl of FACS buffer and resuspended in 150µl FACS staining buffer. Cells were placed on ice in the dark and normally read within 1-2h. For longer term storage, cells are transferred to FACS buffer containing 1%PFA and can be kept up to one week in the dark at 4°C. FlowJo software was used to analyze the data.

For phage binding, cells were washed with PBS and 2ml of accutase as added to the cells for 5min. Cells were triturated to obtain single cells and centrifuged at 900rpm for 5min. Cell pellet was resuspended in FACS buffer and filtered though 40µm mesh to give single cells and small clumps containing up to around 10 cells. The cell concentration is adjusted to 2.5E6cells/ml in order to add 100µl cells per well in a polypropylene 96 well V-shape plate. Then 100µl of phage of interest diluted in FACS buffer is added and incubated for 2h on ice. Cells were washed 3 times with FACS buffer and the primary antibody was added and incubated on ice for 2h. Cells were again washed 3 times with FB and the anti-phage M13 antibody was added and incubated for one hour on ice. Cells were washed 3 times with FB and the secondary antibody was added (usually at a 1:200 dilution) and incubated 30min on ice. Cells were suspended in FB containing 4% PFA to fix the cells and stored at 4°C until FACS analysis (O/N maximum).
7.3. Detection and quantification of human IgG by ELISA

ELISA washing and dilution buffer (EWB) was composed from 25mM Tris pH 7.5, 150mM NaCl, 0.05% Tween® 20 and 0.1% BSA. A Maxisorb plate was coated with 2µg/ml goat anti-Human IgG, (Fcγ-specific) diluted in TBS and incubated overnight at 4°C. The plate was washed twice with EWB and was blocked for 1h at 37°C with TBS+2% BSA. The plate was washed three times with EWB and the antibody (sample or calibration standards) was added to the well and the plate was incubated 1h at 37°C. The plate was washed three times with EWB. The detection antibody (Anti-Human kappa light chain peroxidase conjugated) was diluted to 1:2000 in 2% BSA and added into the well for 30min at RT. The plate was washed three times with EWB and binding was detected by adding 100µl TMB peroxidase substrate. The plate was incubated for 10min in the dark at RT and the reaction was blocked by the addition same volume of the 2N sulfuric acid. The plate was placed in a plate reader and read at OD_{650}.

7.4. Immunohistochemistry (IHC) and Immunocytochemistry (ICC)

7.4.1. Tissue preparation and sectionning

Transcardiac perfusion: Transcardiac perfusion is used to remove blood from the body. Blood can be replaced by buffer, saline solution or fixative. PFA and PBS solutions used for perfusion were prepared freshly under the hood. After lethal anesthesia (§4.2.2), the rat was attached on a cork board. The thorax was rapidly opened to expose the heart and major vessels. The heart was held with a forceps, and the tip of the heart was cut off with sharp scissors. A canula was inserted through the left ventricle into the ascending aorta and clamped with forceps, and the perfusion pump was started at a flow rate of 50ml/min. At the same time, the right atrium was opened with a sharp hook to allow blood to escape. For fixation, the perfusion solution is switched after 2min from PBS to PFA and perfusion continued for a further 10min.

After perfusion, the tissue of interest was rapidly dissected and postfixed in the fixative for 4h at 4°C. The tissue was finally rinsed in PBS. If the block was frozen, fixed tissues were cut with a sliding microtome or with a vibratome. If the tissue was fixed but not frozen it was cut with a vibratome without cryoprotection.
7.4.2. Detection with fluorescence labelled antibodies

Cells were grown to confluence in 96 well plates. The volume of reagent added for the immunocytochemistry (about 500µl/cm²) was sufficient to cover completely the cells. All the washes and incubations were performed on an orbital shaker at 250rpm. Cells were washed with 1X PBS and freshly prepared 4% PFA was added and incubated for 15min at RT. Cells were washed once with 1X PBS and blocking buffer (5% BSA+ 2% milk in PBS filtered through 100µM filter) was added and incubated for 1h at RT. The first antibody diluted in antibody buffer (PBS+5% BSA) was added and incubated O/N at 4°C. Cells were then washed three times with PBS+ 0.05% Tween® 20. Antibody buffer containing second antibody linked to the Alexa Fluor 488 or to Cy5, and supplemented with Hoechst diluted 1:2000 was added and incubated for 1h at RT. Cells were washed three times with PBS, 0.05% Tween® 20 follow by one final wash with 1X PBS. Cells were stored in the dark, and observed on an inverted fluorescent microscope.

7.4.3. Detection with immunoperoxidase staining (DAB-ABC-Method)

Elite ABC kit from Vector Laboratories was used. Tissue sections were rinsed PBS in 12 wells culture plates and endogenous peroxidases were blocked in PBS containing 0.6% H₂O₂ for 10min at RT and washed with 1X TBS 3 times 5min. Section were incubated O/N at 4°C in the primary antibody diluted in 1X PBS pH7.4, 0.3% Triton X-100, 2% Normal goat serum (NGS) (since all our biotinylated secondary antibody are raised in goat). Section were washed 3 times 10min in PBS and then incubated 1h at RT in the secondary antibody solution (biotinylated antibody diluted at 1:500 in PBS pH7.4, 0.3% Triton X-100, 2% NGS). Sections were washed 3 times 10min in PBS and then incubated 1h at RT in the Avidin-Peroxidase (ABC)-Solution (from Vectastain Elite kit standard) prepared by mixing 0.5% reagent A and 0.5% reagent B in PBS pH7.4 at least 30min prior to use. Sections were washed 3 times 10min in Tris/HCl pH7.4, Triton X-100. The reaction was started by adding DAB-H₂O₂ solution (H₂O₂ was added in order to catalyze the reaction) and when the appropriate colour was obtained the reaction was stopped by transferring the sections into PBS. The reaction should not exceed 15 to 30min. Sections were washed twice in PBS, mounted on gelatinized glass slides and left to dry at RT or in oven (30min, 55°C). Slides were dehydrated by dipping them successively into 70% ethanol for 5min, 96% ethanol for 5min, 100% ethanol for 5min, xylene for 3min, 3 times. Finally, slides were mounted in Eukitt.
RESULTS

1. Choice of the controls

Up to now, few protein-based controls and no phage-based controls exist to study the transport of molecules from the blood into the CSF. Thus, the first stage of this work has focused on developing a molecule which could be used as a positive control for our experiments. From the literature we have identified several possible receptors which should be present on the CP and whose ligands may be transcytosed. Among these we chose to explore, the receptors for transferrin, EGF, erythropoietin, leptin and the multiple cargo receptor, LPR2.

We decided to test three types of control phage expressing (i) an antibody fragment (scFv against the anti-rat transferrin receptor, OX-26), (ii) candidate receptor ligands (hErythropoietin, rLeptin, rEpidermal growth factor) and (iii) peptide ligands (angiopep, Arginine vasopressin). All these molecules have been described in the literature as ligands for receptor mediated transcytosis and thus could potentially serve as transporter controls. We will describe here only one example of the construction and characterization for each type of control: a peptide (§1.3.1), a protein (§1.3.2) and an antibody fragment (§1.3.3).

1.1. Identification of known RMTs on CP

In order to determine which control proteins will be chosen, the presence of their respective receptors has been tested by RT-PCR on the CP and on the Z310 cell line. RNA was extracted from both types of sample and RT-PCR was performed using the gene specific primers described in the appendix.
Figure 48: Presence of the receptors was confirmed by RT-PCR

Fig. A: Choroid Plexus Tissue, Fig. B: Z310 CPe cell line

Predicted size: TfR (518bp), EGF-R (653bp), EPO-R (585bp), Leptin-R (556bp), LIF-R (595bp), AVP-R (630bp)

As shown in the Figure 48, in all cases we observed a major band of the predicted size in both samples. All bands were excised from the gel and sequenced in order to verify the identity of the PCR product and all sequences were as expected (data not shown), confirming the presence of each receptor both in the tissue and in the Z310 cell line. In a separate experiment the presence of LRP2 was also confirmed by RT-PCR in CP tissue (data not shown).

The presence of all receptors in Z310 cells increased our confidence that these cells could also be used for transport experiments using the appropriate ligands. Presence of functional LRP2 receptor protein was also tested on Z310 cells by FACS using the rabbit anti-LRP2 polyclonal antibody H-245 which targets the cytoplasmic domain of LRP2. HEK293 kidney derived cells are known to express LRP2 receptor and were used as positive control. Labeling of the cells with the second antibody alone (anti-rabbit PE) was used as a negative control.
Table 15: Binding of anti-LRP2 antibody detected by FACS

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Cells (%)</th>
<th>Z310</th>
<th>HEK293</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit pAb Anti-LRP2 H-245</td>
<td>13.8%</td>
<td>7.6%</td>
<td></td>
</tr>
<tr>
<td>Anti Goat PE Linked (Control)</td>
<td>1.7%</td>
<td>0.3%</td>
<td></td>
</tr>
<tr>
<td>Anti Rabbit PE Linked (Control)</td>
<td>1%</td>
<td>0.1%</td>
<td></td>
</tr>
</tbody>
</table>

Percentage of positive cells; Data from the FACS were analysed with the FlowJo software and are reported as geometric mean from the canal FL1-H

Labelling with anti-LRP2 shows specific binding to both Z310 and HEK293 cells confirming the presence of LRP2 on the surface of Z310 cells.

Thus from these data we conclude that the CP and CPe derived cell line Z310, both express the receptors we wish to target with our control phage.

1.2. Anti-rat transferrin receptor antibody, OX-26

The antibody OX-26 is known to bind to the rat transferrin receptor (TfR). It appears to be transported across the BBB, and several groups have investigated the potential of OX-26 as a brain transport vector (Introduction part §6.4). As our results have shown in the §1.1 TfR is also present in the choroid plexus epithelium, and we therefore selected this antibody as a peptidomimetic positive control in our study of the BCSFB.

Cloning strategy the OX-26 V\text{H} and V\text{L} and production of recombinant phage

OX-26 is a mouse IgG2a but the DNA sequence of the heavy and light chain variable regions was not available. We therefore undertook to clone these regions and for this the following strategy was adopted:
- Purification of the commercially available OX-26 antibody from the hybridoma (material and methods part §2.4.2 and §2.6).
- N-terminal sequencing of the antibody heavy and light chains (§1.2.1).
- Design of degenerate 5’ PCR primers able to recognize all possible codons encoding the N-termini of the heavy and light chain.
- RT-PCR using total RNA from the OX-26 hybridoma as template, and primer sets comprising the 5’ degenerate primer mix, together with 3’ primers based on the known sequences of the mouse IgG2a heavy and light chain constant regions.
- Purification and sequence confirmation of the DNA fragments encoding the V_H and V_L regions.
- And finally subcloning into the phagemid vector pHal14 in both the V_H-V_L and the V_L-V_H orientations.
This is described in more details below.

1.2.1. N-Sequencing of OX-26
Production and purification of OX-26 from the hybridoma is described in the material and method part §2.4.2. The light and heavy chain bands were separated by SDS-PAGE and subjected to N-terminal sequencing using our internal amino acid sequencing service. Results presented in Table 16 show the first 9 amino acids from the heavy chain and the first 10 amino acids of the light chain with ambiguities as indicated.

<table>
<thead>
<tr>
<th>OX-26 HC</th>
<th>Amino acid</th>
<th>Mouse CH1 V00825  IGHG2A*01</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>immunoglobulin heavy constant gamma 2A</td>
</tr>
<tr>
<td>Type</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Q V Q L Q E P G A Q</td>
<td></td>
</tr>
<tr>
<td>OX-26 LC</td>
<td>Amino acid</td>
<td>Mouse IGKC V00807  IGKC*01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>immunoglobulin light constant kappa 2A</td>
</tr>
<tr>
<td>Type</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>D I Q M N Q S S S S L P</td>
<td></td>
</tr>
</tbody>
</table>

Table 16: Amino acid N-sequencing results

The cloning of V_L OX-26 and V_H OX-26 from OX-26 hybridoma cells into pBluescript vector is fully described and documented in the material and methods §1.5.1. The degenerate primers used to amplify the OX-26 heavy and light chain variable regions are based on the N-terminal protein sequences and are shown below (Table 17).
Table 17: Sequence of the degenerate primers for the OX-26 variable regions

Bottom strand primer was based on the known sequence of the constant region. The figures in brackets show the level of degeneracy in each case.

The strategy adopted to obtain the scFv is shown in Figure 33 in the material and methods section.

To confirm that we had cloned the functional OX-26 antibody, the variable regions were also reformatted as an normal IgG as described in Materials and Methods (§2.6) and tested as described below.

1.2.2. Binding of hybridoma OX-26 and recombinant OX-26 to Z310 cells as tested by FACS

In this experiment we have compared the binding of the antibodies purified from the OX-26 hybridoma and the recombinant reformatted antibody for their ability to bind to Z310 cells which express the transferrin receptor. Antibodies were purified as described in material and methods §2.6, and Z310 cells were prepared for FACS following detachment with accutase instead of trypsin. Cells were incubated with increasing concentrations of the OX-26 antibodies from 0 to 10µg/ml. Goat-anti-mouse IgG(Fc)-Alexa Fluor 488 was used as second antibody. The results are presented in Figure 49.
**Figure 49: Binding of OX-26 detected by FACS**

A. Dose response of hybridoma OX-26 on Z310 cells measured by FACS. B. Detection of the binding of recombinant OX-26 on Z310 cells detected with goat anti-mouse AF488 linked.

In both cases, addition of increasing concentrations of OX-26 gave a similar shift in mean fluorescence intensity (MFI) while the mouse IgG2a isotype control antibody for the rat hybridoma derived OX-26, and the human IgG control for the recombinant OX-26 gave no signal even at the highest concentration tested (10µg/ml). These results confirm the functional binding of our recombinant OX-26 antibody. It should be noted that for the recombinant OX-26, the variable regions were reformatted in the human IgG framework since this is the format in which our candidate antibodies will be produced.
1.2.3. **Testing the binding of recombinant OX-26 by ICC**

Having demonstrated that recOX-26 binds to Z310 by FACS analysis we wanted to investigate the subcellular localisation of this binding (e.g. intracellular or membranous). For this, Z310 cells were grown and fixed with PFA (material and methods part §7.4) and incubated with OX-26 antibody or with an irrelevant antibody in the same framework. After washing, the presence of antibody was detected using a Goat anti Human-Fc-cy5 secondary antibody. Nuclei were stained with Hoechst dye and cells were observed using an inverted microscope.

![Image](image.png)

**Figure 50: Binding of rec OX-26 to Z310 cells determined by ICC**

**Fig. A:** Binding of rec OX-26 at 20µg/ml on fixed Z310 cells. **Fig. B:** ICC of human irrelevant antibody at 20µg/ml on fixed Z310 cells.

As shown in the Figure 50, rec OX-26 antibody shows strong binding to Z310 cells compared to the irrelevant antibody, and labelling of the transferrin receptor is clustered into discrete foci in keeping with its localization into clathrin coated vesicles\(^{124}\). This confirms the correct refolding and activity of the recombinant OX-26 mAb.

1.2.4. **Test of binding of the scFv OX-26 phage to Z310 cells**

The aim of this experiment was to test whether scFv phage displaying the OX-26-PIII fusion protein could be used to detect the TfR on Z310 cells even though phage are notoriously sticky when used in different immunological procedures. Wild-type phage were used as a negative control (empty phage).
For these experiments Z310 cells were grown on collagen I coated 24 well plates to improve adherence, and fixed with 5% PFA at confluence. Non specific binding was blocked with BSA/milk solution.

Increasing numbers of each phage (400µl/well containing 10e12, 10e11, 10e10, 10e9, 10e8 or 0 cfu/ml) were added to the fixed cells.

Unfortunately, strong binding was found in all cases and no increased signal of the OX-26 phage compared to empty phage could be detected even when the concentration of phage was decreased to 10e8 cfu/ml (data are not shown). The lack of specific binding in this experiment is probably due to the inherent 'stickiness' of phage as a probe for ICC, and this problem has never been successfully resolved (Steven Dunn personnal communication).

Since we were not able to show specific binding of the OX-26 scFv by immunofluorescence using fixed cells, we decided to test the binding to Z310 cells by FACS.

To test the OX-26 phage by FACS, Z310 cells were prepared for FACS experiment as described (material and methods §7.2). 1E11 phage were incubated 2h on ice with the Z310 cells and phage were detected with the polyclonal anti M13 antibody followed by a secondary antibody linked to Alexa Fluor 488. The results are shown in Figure 51.

![Figure 51: Representative figures of the FACS experiment to determine the binding of scFv OX-26 phage](image_url)

The OX-26 phage preparation was a 1:1 mix of scFv phage expressing OX-26 V\textsubscript{L}-V\textsubscript{H} and OX-26 V\textsubscript{H}-V\textsubscript{L}. Irrelevant phage are against a human protein not cross-reactive in rat. The results presented were obtained using 1E11 cfu/ml concentration.
In this experiment we observed a strong signal in the presence of OX-26 phage compared to the negative control phage expressing irrelevant antibody or the second antibody alone. The high background signal observed in the presence of non specific irrelevant phage is again probably due to inherent phage ‘stickiness’ which is known to complicate different immunological approaches; nevertheless a specific binding with the scFv phage to the TfR could be detected above the control phage signal, confirming the correct folding of the OX-26 antibody in the scFv format.

In summary, using the commercially available OX-26 hybridoma, we were able to determine the sequences of the heavy and the light chain of the anti-TfR antibody and using this information we were able to clone the OX-26 heavy and light chain variable regions. The antibody was reformatted into a human IgG framework and we confirmed that this new antibody has a similar binding to the rat transferrin receptor as seen for the commercial OX-26 antibody. The variable domains of the heavy and light chains were subsequently assembled into the pHal14 phagemid vector for expression as a scFv fragment fused to the G3p protein on the phage surface.

1.3. Construction of additional phage controls

Given that there were no well-established positive controls for our BCSFB transport experiments, we decided to clone the cDNAs for some additional proteins and peptides to test using our in vivo phage display approach. All the selected proteins were first cloned into an expression vector and then engineered into the pHal14 phagemid vector in order to display the protein on the surface of a phage. In all, 12 different phagemids were constructed and corresponding phage with different ligands fused to the G3p were expressed on the phage surface.
<table>
<thead>
<tr>
<th>Description</th>
<th>Vector</th>
<th>mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angiopep-gIII fusio</td>
<td>pHal14</td>
<td>0.26</td>
</tr>
<tr>
<td>empty vector (pelB-gIII)</td>
<td>pHal14</td>
<td>0.43</td>
</tr>
<tr>
<td>RAP-full length-gIII fusion</td>
<td>pHal14</td>
<td>0.36</td>
</tr>
<tr>
<td>RAP-domain 3-gIII fusion</td>
<td>pHal14</td>
<td>0.35</td>
</tr>
<tr>
<td>hErythropoietin-gIII fusion</td>
<td>pHal14</td>
<td>0.28</td>
</tr>
<tr>
<td>rLeptin-gIII fusion</td>
<td>pHal14</td>
<td>0.22</td>
</tr>
<tr>
<td>hLIF-gIII fusion</td>
<td>pHal14</td>
<td>0.18</td>
</tr>
<tr>
<td>DSIP-gIII fusion</td>
<td>pHal14</td>
<td>0.26</td>
</tr>
<tr>
<td>Arginine Vasopressin-gIII fusion</td>
<td>pHal14</td>
<td>0.28</td>
</tr>
<tr>
<td>OX-26_VL-VL</td>
<td>pHal14</td>
<td></td>
</tr>
<tr>
<td>OX-26_VL-L</td>
<td>pHal14</td>
<td></td>
</tr>
<tr>
<td>EGF</td>
<td>pHal14</td>
<td></td>
</tr>
</tbody>
</table>

**Table 18: List of the control phagemid produced**

Glycerol stocks from all phagemids were prepared. All the primers used for RT-PCR and PCR for the other controls were described in the appendix part.

Recombinant phage were rescued with hyperphage and purified using the Apanovis kit as described in (material and methods §3.2.4). For in vivo injection, endotoxins were depleted using Triton X114 (material and methods §3.2).

By way of illustration, in the following 3 sub-paragraphs, we briefly describe the cloning of one peptide control (Angiopep), of one protein control (rat Leptin) and one of sdAb antibody fragments, (FC5). Rescue and amplification of the phage in each case was performed as described in the material and methods §3.2.

### 1.3.1. Cloning of the Angiopep peptide

Angiopep is a 19 amino acid peptide (TFFYGGSRGKRNNFKTEEY) based on similarity to the Kunitz domain which binds to LRP and LRP2 and which is being developed commercially\(^\text{125}\). As this peptide is small, we have designed 2 complementary oligonucleotides (MS1149 and MS1150) which fully encode the above sequence with appropriate restriction sites (Ncol and BamHI) for cloning into the pHAL14 phagemid vector. The two oligonucleotides were allowed to anneal and the resulting 80bp fragment encoding the Angiopep insert was digested with Ncol and BamHI and gel purified. The pHAL14 vector was processed in parallel (Figure 52).
After ligation and transformation of E. coli, single colonies were picked, and the plasmid DNA was extracted and sequenced. A clone encoding the correct Angiopep sequence in frame with the phage G3p was retained for phage production.

1.3.2. Cloning of rat leptin

Rat leptin is 167 amino acids in length and the 520bp cDNA was cloned by RT-PCR. using the RT Primer MS930 and the PCR amplification primers MS931 and MS932.
As shown in the Figure 53, a band was visible between 500bp and 600bp at the expected size for rat Leptin of 520bp. This fragment was digested with appropriate restriction enzymes, purified and ligated into the pHal14 vector and the correct sequence of rLeptin was confirmed by DNA sequencing (Appendix part).

1.3.3. **Cloning of one antibody fragment control: FC5**

As described in the introduction, FC5 is a single domain antibody (sdAb) fragment selected from a llama library for its ability to translocate through the BBB\(^{102}\). The sdAb contains only the variable heavy region \(V_{H}\). The DNA sequence shown in Figure 54 was synthesized at Geneart with NcoI and NotI sites at the 5' and 3' ends respectively underlined.

```plaintext
CCATGGCAGAGGTCAGCTGCAGGGCTCTGGAGGGATTGTGCGAGGGCTGGGCTCTGAGACT
CTCTGTGCAGCTCTGTGATTCAAATCAGCTATATACATTGCGGTGTCGCTTTCCGGGCTCCAGGG
AAGGAGCGTGAAATGTATACGATATTACTTGTTGTTGATAAATACCTTCTATTCAAACGTG
AGGCGCGATTCACCATTTCCGAGACAACGCCAAGGAGGACTCTCTATTGAGTCGAGGACTCTTG
GACTACTGGGGCAAGGGAGCCAGCCAGGCAGGCTGTGCACTGCACTGACGAGGACTCTTG
GACTACTGGGGCAAGGGAGCCAGCCAGGCAGGCTGTGCACTGCACTGACGAGGACTCTTG
```

Figure 54: DNA sequence of the sdAb FC5

Lama glama immunoglobulin heavy chain variable domain FC5 (sdAb) mRNA, partial cds Accession number Genbank AF441486, The **underlining bold characters** represent the NcoI restriction site and the **underlined italic characters** represent the NotI site.

The cDNA was amplified by PCR, digested with NcoI and NotI. The 428bp band (Figure 55B) was gel purified for ligation into the pHal14 phagemid vector and transformation into E. coli, TG1. Phagemids from individual colonies were sequenced and the FC5 insert was confirmed. One clone was selected and used for phage production using the hyperphage rescue procedure described in the methods section (§3.2.3).
1.4. Low density lipoprotein-Related Protein 2 (LRP2)

Sequence analysis and comparison Kidney and Choroid Plexus

The 521kDa cargo receptor LRP2 has many ligands and has been proposed as a potential route into the brain. However, while LRP2 is present in the BBB and on the CPe it is far more abundant in the kidney which would clearly be an issue for any therapeutic applications. With this in mind, we were interested to find out whether different isoforms may be differentially located in the kidney and brain and whether such differences could provide a more specific route for targeting the brain.

The primary aim therefore was to compare the full sequence of LRP2 in the two different tissues, kidney and CP. For the purpose of this experiment, the 15438bp sequence of LRP2 has been virtually divided into 2000bp sections in order to be able to sequence each section. Sequencing primers have been designed and ordered for each section (full sequence in the appendix part).

The RNA extraction, cloning and sequencing were performed for LRP2 from the kidney and from the CP as described in the material and methods section.
The sequences obtained from the brain and kidney LRP2s were aligned and compared using ‘in-house’ informatic tools but no difference between CP-LRP2 and kidney-LRP2 was detected. In the absence of a brain specific isoform of LRP2, this strategy was not pursued any further.

2. *In vivo* model

2.1. Set up of the *in vivo* phage display

2.1.1. Phage injection

The phage library has a complexity of about 2.9E9 phage. Thus in order to have multiple representation of each clone (on average about 30x), we have injected ~1E11 phage/animal for the first round of panning. For the second and third rounds of panning, where the phage diversity is expected to be reduced following the first round of selection, we have injected 1E9 phage/animal. For the preliminary set-up experiments, the control phage described above were used.

To inject phage into the blood, we had the choice between intra-arterial (carotid artery) or intra-venous (tail vein) injection. The intra-arterial route has the advantage that phage are transported directly into the brain, however for our panning experiments we reasoned that intra-venous injection would be more suitable, firstly because the tail vein is easily accessible in the rat, and second, this route allows the phage to circulate through the body before reaching the brain, and thus most of the non-specific phage binding will be eliminated through negative selection in the peripheral tissues prior to reaching the brain. In rats, volumes of up to 0.5ml can be injected into the tail vein, which is sufficient to inject the full phage library at its initial concentration of 1.15E12 cfu/ml.

For the phage library, we have used the scFv library from Stefan Dubel\textsuperscript{100} which was rescued using phage which were deleted for gene III (hyperphage). In this library, all copies of gene III on the phage surface are recombinant and display a scFv-G3p fusion protein.
2.1.2. Preparation of the phage for injection: elimination of LPS

A major problem in working with bacteriophage \textit{in vivo} is the presence of toxic lipopolysaccharides (LPS) produced by the \textit{E. coli} host during phage preparation, and which could lead to rapid septic shock when injected into animals.

The limit fixed by the FDA for human injection is 5EU/kg of body weight. For safe injection into animal, a maximum of 50EU/ml LPS is recommended for the injection of new biological entities (internal guideline from Merck Serono) whereas the LPS content of a typical phage pool isolated directly from \textit{E. coli} is greater than $10^6$ EU/ml.

To overcome this problem we have developed a procedure to remove LPS based on sequential extraction with the detergent, Triton-X114 which has a cloud point of 23°C. The procedure is described in detail in the methods section §3.2.5.

In the Figure 56, we have measured the LPS concentration after each round of LPS extraction. From an initial concentration of about 8E5 EU/ml of LPS, we can see that 4 rounds of LPS extraction are sufficient to reduce the level of LPS below 50EU/ml which is the acceptable limit for \textit{in vivo} injection. In our routine production of phage, we have decided to perform 6 cycles of LPS depletion to ensure that we reach this limit.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{lps_depletion.png}
\caption{Concentration of LPS in the phage sample after increasing number of LPS extraction}
\end{figure}

Experiment repeated several times. The shown data are representative from one single experiment.
2.1.3. Effect of heparinated blood, serum and plasma, on phage infectivity

Mammalian blood is not a normal environment for phage, so we needed to establish that exposure to enzymes and other factors in blood would not interfere with the subsequent phage recovery and quantification. In the experiment shown in Figure 57, we have tested the effect of different environments on phage infectivity of the natural E. coli host.

![Figure 57: Influence of the sample type on phage infectivity](image)

As shown in Figure 57, the incubation of phage in plasma, serum or whole (heparinated) blood for up to 22h does not affect subsequent infectivity and recovery through E.coli.

2.1.4. Time course for in vivo panning

CSF has a high turnover. It is completely replaced 4-5 times per day and is recycled back into the blood via the subarachnoid space and the sagittal sinus. In rat, the total volume of the CSF is about 100µl. Thus a phage which has the ability to cross the BCSFB may not remain free in the CSF for a long before either binding to its specific target or being recycled into the blood. This suggests that to recover phage transported into the CSF,
samples should ideally be taken at different time points during the first few hours following the injection. In our final protocol (see below) CSF was collected at 15min, 1h and 2h after the phage injection.

2.2. 

*In vivo* phage display and biopanning results

2.2.1. The initial strategy

During the set-up phase to optimize the procedures for *in vivo* phage display, experiments were performed using a pool of control phage developed and constructed as described in the §1. Our initial strategy was to inject phage into the peripheral blood via the tail vein and to recover the CSF to identify any transported phage.

For this, a mixture containing equal numbers of 9 control phage was prepared in PBS at a final concentration of 1E12cfu/ml. 0.5ml of the mix was injected intravenously into 3 adult rats and left for 1h in order to allow phage to circulate in body and distribute into tissues. After deep anaesthesia with pentobarbital, CSF was sampled directly from the cisterna-magna using a 31G insulin syringe, and a sample of blood was collected in heparin microtubes by intra-cardiac puncture. Phage were recovered from serum and CSF by infecting bacteria and 96 colonies of each were picked for plasmidic DNA extraction and sequencing.

![Figure 58: First *in vivo* phage display experiment](image)

Empty vector: phage containing only the PIII protein and consider as negative control. Phage considered as potential positive controls: Angiopep, RAP-FL: RAP full length, RAP-D3: Domain 3 of RAP, hEPO: human erythropoietin, rLeptin: rat leptin, hLIF: human LIF, DSIP, AVP
Results are presented in the Figure 58. Disappointingly, we found no statistical difference between the proportion of each clone present in the CSF compared to the proportion in the serum whereas we could have expected a reduced representation of empty phage in CSF and an increased representation of positive translocated clones in the CSF.

In fact these results were highly informative insofar as they illustrated the difficulty, and the importance, of completely eliminating all traces of contaminating blood when sampling CSF. Clearly given the high concentration of phage injected into the blood and the low numbers expected to be recovered in the CSF, even the trace amount of blood introduced into the sample following puncture of the cisterna magna are sufficient to mask any actively transported molecules. The same results were observed in two further experiments even taking care to reduce blood contamination to a minimum.

We therefore invested considerable effort in finding other ways to sample CSF more cleanly.

2.2.2. Quantification of the level of blood contamination in CSF
Several sensitive methods to monitor the blood contamination were considered, such as the measurement of the concentration of haemoglobin or blood enzymes however such procedures were not possible because of the limiting volumes of CSF recovered. More useful may have been to use radiolabelled tracer molecules, however injection of radioactive molecules into rats was not permitted with our OVC authorisation. In the end we opted for a very simple test consisting of centrifuging the CSF at 6000rpm for 30sec in order to pellet all the blood cells. The supernatant was the recovered for phage rescue, while the pellet was resuspended into 5µl of PBS and red blood cells were counted under the microscope. With this quick test, we were able to monitor and quantify even very low levels of blood contamination with high accuracy and without consuming the CSF sample.

2.2.3. Approaches to optimise CSF sampling
The method routinely used for CSF sampling from the cisterna magna has been described in the material and methods §4.2.5. With this method, the needle penetrates through the skin and the muscles and as a consequence minor blood contamination of the CSF inevitably occurs.
To improve the procedure in order to meet our stringent experimental requirements several approaches were tested.

First we tried extensive whole animal perfusion with PBS in order to remove all blood from the brain prior to CSF recovery; however this was not successful since the perfusion resulted in loss of pressure in the ventricular system and collapse of the cisterna magna.

A second approach was to very carefully dissect the rat in order to expose the cisterna magna. This was done with an animal fixed on a stereotaxic instrument as shown in the Figure 59. With scalpel and forceps, surrounding tissues were first removed to expose the arachnoid membrane and a butterfly needle with a clampable catheter was used to exclude blood contamination at the beginning and end of the sampling process. This procedure gave some good results but was too delicate and time consuming to be used for our routine sampling especially at early time points.

![Image: Dissection in order to expose arachnoid membrane delimiting the cisterna magna](image)

**Figure 59: Dissection in order to expose arachnoid membrane delimiting the cisterna magna**

**Fig. A:** Exposure of the cisterna magna (view from the top). **Fig. B:** Explanation of the butterfly needle with clamps on both ends in order to stop a visible blood contamination.

We also attempted to sample CSF directly from the cerebral ventricles using the stereotaxic instrument, however while the ICV route is suitable for injection into CSF, withdrawal from it by aspiration causes rapid collapse of the ventricles.

After different tests the final procedure adopted for this study was based on long-term canulation of the cisterna magna using a rat model recently available from Charles Rivers.
Laboratories. For this type of experiment, a new authorization from the OVC (veterinary authority) was obtained.

Rats were received with a guide canula inserted through the skull into the cisterna magna as described in the Figure 60. Surgery was performed about 1 week before reception of the animals allowing scar tissue to form and seal the wound. The canula was closed by a protective screw cap. After reception, rats needed to be stabilized in the animal facility for one week before starting the experiment.

To sample CSF, the screw cap was removed and a specially designed internal canula was inserted which extends about 1mm beyond the end of the guide canula (Figure 70). CSF was recovered using either the natural intraventricular pressure or by very slight negative pressure applied with a syringe. Using this procedure, a volume of 30µl samples of CSF could be withdrawn easily and the sampling could be repeated at different time intervals.

![Figure 60: Representation of the guide canula inserted into the cisterna magna](image)

The final protocol used to harvest CSF was as follows: Animals were warmed to 39°C in a warm cabinet for 10min to dilate the tail vein, and 0.5ml of the LPS-depleted phage was injected using a 25 gauge needle. At 15min, 2h, 5h and 24h, 30µl of CSF was withdrawn and centrifuged to confirm the absence of blood cells as described above. In parallel, approximately 50µl peripheral blood was taken from a small incision into the saphen vein. Phage were recovered from both samples for further analysis.
2.2.4. Recovery of phage from CSF using the long-term canulation model

1.4.3.1. First round of in vivo panning

10^{11} hyperphage from the Dübel library were injected into 2 rats and CSF was recovered at different time points (15min, 2h, 5h, 24h). Quality of the CSF was very good and no red blood cells were detectable in the pellet after centrifugation. After bacteria infection, about 10 000 colonies were present in the 15min sample and lawn of colonies were visible on the other conditions.

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>HV CDR1</th>
<th>HV CDR2</th>
<th>HV CDR3</th>
<th>LV CDR1</th>
<th>LV CDR2</th>
<th>LV CDR3</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSS-PL303/06_H12_2/GTFT--S-SSYA</td>
<td>ISD--G-GETT</td>
<td>ARGQT--------GNPV</td>
<td>NG--SGS</td>
<td>DO--S</td>
<td>QTYACOS</td>
<td>SDFMV</td>
</tr>
<tr>
<td>SSS-PL303/06_E13_2/GTFT--S-SCSY</td>
<td>ISD--G-GETT</td>
<td>ARGQT--------GNPV</td>
<td>RMND--GNVY</td>
<td>EG--S</td>
<td>CTSKCG</td>
<td>SSTL</td>
</tr>
<tr>
<td>SSS-PL303/06_E8_2/GTFT--S-TSYD</td>
<td>MNPN-GGHT</td>
<td>ARNNSSY-------YSLOD</td>
<td>STS--ASNY</td>
<td>ED--N</td>
<td>QSYDGSSWCGS</td>
<td>ARGPSPP8</td>
</tr>
<tr>
<td>SSS-PL303/06_A15_2/GTFT--S-TSYY</td>
<td>INPM-GGHT</td>
<td>ARNNSSY-------YSLOD</td>
<td>SNNV--GNQ</td>
<td>RR--N</td>
<td>QTVGTS</td>
<td>GSVV</td>
</tr>
</tbody>
</table>

Table 19: Candidates from the round 1 output with canulated rats.

To obtain an initial impression of the performance of the long-term canulation model, 2 x 96 colonies from the 15min output were picked for sequence analysis. Analysis of these data showed that 129 clones (70%) were full length and in frame with the G3p signal sequence, and interestingly, in this pilot analysis, 2 pairs of clones were obtained with the same HV-CDR3 and related HV-CDR1 and CDR2, but with totally different LVs (Table 19). Since the heavy chain CDR3 is generally thought to be a major determinant in antibody specificity, these 4 clones were considered as potential candidates.

All the colonies from each time point of the round 1 output were pooled separately and phage were rescued using helper phage. Rescued phage were prepared and titered ready for injection in round 2.

1.4.3.2. Second and third round of in vivo panning on canulated rats

One rat was injected with 10^{11} phage from the round 1 15min output and a second rat was injected with the round 1 output from the combined 2h and 5h outputs. We decided to study the 15min output separately in the hope of finding more specific and more highly reactive phage ligands. In round 2, CSF was again sampled at 4 different times points (15min, 1h, 2h, 5h) and clean CSFs were recovered in all cases and used for bacterial infection. 96 colonies were picked from each condition. After sequence analysis, we found that only 26 clones (5.3%) from about 600 sequenced clones were in frame with the scFv signal
sequence (data not shown). Nevertheless we have decided to continue for a third round of \textit{in vivo} panning.

For the third round of panning, $10^{11}$ phage from round 2 output were injected in the tail vein of a rat. CSF and plasma were sampled at 15min, 2h and 5h. After bacterial infection, about 30 000 colonies were recovered and 348 colonies were sequence from the 15min and pooled 2h and 5h samples as above. Disappointingly, the sequence analysis showed that the proportion of mutated clones was increased even further than in round 2 and only 3% of clones sequenced were intact and in frame with the G3p signal sequence.

As explain in the discussion section, this was a problem of using helperphage instead of hyperphage. Indeed hyperphage are polyvalent phage with a better avidity due to there increased number of displayed molecules.

2.2.5. \textbf{Strategy using canulated rat and polyvalent phage rescue using hyperphage}

Phage from the round 1 output described above (§1.4.3.1) were used, and the 15min, 2h and 5h samples were rescued separately using the hyperPhage M13K07 ΔpIII as described in the material and methods (§3.2.3). Phage were purified, LPS depleted with Triton X-114, and titered for the round 2 injection into the canulated rats. For the round 2 input, $10^{10}$ phage were injected into the tail vein of each rat.

A total of 260 colonies were recovered from the CSF sampled at 15min, 2h and 5h while the phage concentration in the blood was estimated to be around $10^{e6}$ cfu/ml, thus confirming the effectiveness of the canulation model in obtaining clean blood-free CSF. All the colonies from the CSF were sequenced and 67 colonies from the plasma were also sequenced to confirm the phage diversity.

The number of defective clones was not increased compared to the round 1 confirming our hypothesis that the use of hyperphage would allow us to recover more intact clones. The results of the sequence analysis are shown in Table 20.
From this analysis, 5 new clusters of identical clones were identified. All were full length intact sequences and in frame with the the GIII signal sequence. Thus all these clones were considered as potential candidate able to enter the CSF.

2.3. Conclusion on in vivo phage display panning

According to our initial plan, the in vivo screening was only the first part of our project. This part took longer than anticipated due to difficulties we encountered first in overcoming the problem of blood contamination of the CSF, and second in resolving the problem of selecting for mutated phage. Both problems were resolved, but as a result, we were not able to develop two approaches which were initially planned, namely testing of the candidate phage in the in vitro transport system and studies on the soluble scFv fragment secreted from the phage. Instead we decided to proceed directly to the task of reformattting the scFvs into the human IgG framework to allow us to investigate the interaction of our potential candidate antibodies with the CP using the more classical and well established format.
3. Reformating of potential candidates

From the *in vivo* phage display screening, we identified several interesting candidates which wished to investigate further. The following candidates renamed by letters were selected for further analysis.

4 candidates (named A, B, C and D) came from the round 1 output: They have identical HC-CDR3 but different LC. A series of 8 candidates (E, F, G H, I, J, K and L) were identified through an alternative strategy in which we attempted to identify phage which were internalized by the choroid plexus following *in vivo* panning (data not shown). And 5 candidates (M, N, O, P and Q) were recovered from the round 2 output performed on the long term canulated rats following rescue with hyperphage.

3.1. Reformating scFv V\_H and V\_L regions into the pTT vectors for co-expression in HEK cells

All plasmid DNAs from the above candidates were recovered from the frozen stock. PCR amplification of each VH was performed using universal pools of primers (Table 21) and cloned into pTT CH1\_20966 (NcoI, Sall). PCR amplification of each LC was similarly performed using a universal pool of primers and cloned into pTT CL\_20968 (BssHII, Fse).

![Table 21: Primers and pool of primers used for the reformating](image-url)
Table 22: PCR conditions for reformatting

<table>
<thead>
<tr>
<th>Product for PCR</th>
<th>For VL</th>
<th>For VH</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>scFv miniprep (diluted 10 fold)</td>
<td>scFv miniprep (diluted 10 fold)</td>
<td>scFv miniprep (diluted 10 fold)</td>
<td>1.5μl</td>
</tr>
<tr>
<td>Forward Primer</td>
<td>BssHII For Pool</td>
<td>UnilacFOR pool</td>
<td>1μl</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>FseI Rev pool</td>
<td>HJSall_REV pool</td>
<td>1μl</td>
</tr>
<tr>
<td>Q H2O</td>
<td>Q H2O</td>
<td>Q H2O</td>
<td>10μl</td>
</tr>
<tr>
<td>2x Long Amp tac master mix</td>
<td>2x Long Amp tac master mix</td>
<td>2x Long Amp tac master mix</td>
<td>13μl</td>
</tr>
</tbody>
</table>

**Figure 61:** Agarose gel to control the quality of the PCR $V_L$

Expected size 400bp; MW: 100bp molecular weight

**Figure 62:** 1.3% Agarose gel to control the quality of the PCR $V_H$

Expected size 500bp; MW: 100bp molecular weight

All the PCR products were checked on agarose gels, the DNA concentration was measured and confirmed by DNA sequencing.
3.2. Production and purification

50ml suspension cultures of HEK293-6E were co-transfected with 25µg of each pair of LC and HC plasmids. In this cell line the IgGs are secreted into the medium and were purified on protein A resin and desalted by G25 column chromatography. IgGs were concentrated by centrifugation through filter with 30 kDa cutoff. Yields are summarized in Table 23.

![Table 23: Concentration of the reformatted antibody (candidates A to Q)](image)

Of the 17 samples, 6 candidates (B, D, G, H, M and Q) failed to express. The 11 remaining candidates were analysed by SDS PAGE as shown in Figure 63. A second attempt to express the missing candidates was performed and resulted in production of candidate M (data not shown). The 5 other combinations which failed to produce antibodies were not pursued further.
3.3. Western blotting of the candidate antibodies against membrane proteins from Z310 cells

In order to test the binding of the candidate antibodies, membrane proteins were prepared from the Z310 cell line by nitrogen cavitation as described in material and methods §6.2. Protein concentration was determined using the Nanodrop spectrophotometer and aliquots containing 50µg/lane were electrophoresed through 4-12% gradient gels and transferred to PVDF membranes. The results of immunoblotting are shown in Figure 64.

Figure 63: SDS-PAGE stained with coomassie blue to test the purity of the antibody

Figure 64: Test of binding of candidat on Z310 membrane protein extract detected by western Blot

No Ab: No primary antibody; IgG: Irrelevant human antibody; A, C, N, O, P: Candidat antibody; CB: membrane stained with ponceau red as transfert control (representative of all the samples)
The positive control OX-26 antibody labels a major band at about 110kDa as expected. A band at about 100kDa is visible in the negative control as well as in the positive control. The candidat O labelled multiple proteins quite intensely.
3.4. Characterization of the candidate antibodies by IHC on tissue sections from rat brain

Choroid Plexus

Cortex

N

N

O

O

P

P
Figure 65: Brain slices were labeled by immuno-histochemistry using different candidate antibodies and control antibodies.

Two regions of the brain were examined, the Choroid Plexus and the Cortex. The binding of the IgG reformatted antibodies N, O and P was observed as well as the OX-26 antibody for control and HEL as negative control.

As shown in the Figure 65, OX-26 labeling shows association with brain microvessels and also binding to the CP while the negative control antibody HEL in the same human IgG format shows no labelling of the brain. Of the potential candidates, antibody P gave the strongest CP labeling and the candidate O show also some positive signal. The candidate N shows no labeling of the tissue above the background signal. No signal with N, O or P in other regions of the brain was visible above the background signal.
4. *in vitro* models of the BCSFB

4.1. Set up of the choroid plexus primary cells

The choroid plexus is an anatomically distinct organ which surrounds the cerebral ventricles. It can be isolated surgically from the lateral and 4\textsuperscript{th} cerebral ventricles. Dissection of the choroid plexus from the 3\textsuperscript{rd} cerebral ventricle in the rat is more complex and is usually not performed. The procedure for setting up primary cell cultures was adapted from Strazielle\textsuperscript{4}. Based on this original protocol, several steps have been optimized or refined in order to suit the needs of the project. The optimization of several key steps such as coating of the culture plate surface, seeding density and digestion time are described in this section.

4.1.1. Determination of the optimal coating of the well

In the original procedure of Strazielle, wells are coated with laminin to facilitate adherence of the cells. In order to optimize the cell adherence we have tested a series of other coating procedures: Laminin, collagen, a mix 1:1 of laminin and collagen IV, and a solution of BD Matrigel\textsuperscript{®} were all tested at different concentrations from 0 to 10mg/ml.

Matrigel\textsuperscript{®} is a complex mixture which consists of purified extracellular matrix proteins extracted from a mouse sarcoma and the principal components in order of abundance are laminin, collagen IV, heparan sulfate proteoglycans, and nidogen. Several growth factors are also present such as epidermal growth factor, insulin-like growth factor, fibroblast growth factor, TGF-beta or tissue plasminogen activator. The composition of Matrigel\textsuperscript{®} is very close to the natural basal lamina.

From preliminary experiments (data not shown), coating with laminin alone or collagen IV alone gave similar results in terms of attachment and growth of the primary cells while coating with the 1:1 mix of laminin and collagen IV gave a better attachment of the primary cells than the individual components alone. However Matrigel\textsuperscript{®} gave the best attachment of the primary cells and was used for the experiments described in this study. Its derivation from a mouse tumour leads to some variability in composition and properties thus for the work described here a single batch was used and stored in small aliquots at -20\degree C.
Figure 66: Determination of the optimal concentration of Matrigel® for the coating of primary cells

**Fig. A**: 0µg/ml (No Matrigel); **Fig. B**: 0.25 µg/ml (1:40 dilution); **Fig. C**: 0.5µg/ml (1:20 dilution); **Fig. D**: 1µg/ml (1:10 dilution); **Fig. E**: 2µg/ml (1:5 dilution); **Fig. F**: 4µg/ml (1:2.5 dilution); **Fig. G**: 0.5µg/ml (Undiluted).

Observation was performed under microscope (X100) after 5 days of culture.

As shown in the Figure 66, the optimal concentration of Matrigel® chosen was 0.25mg/ml in DMEM/F12 medium (Figure 66B). At high concentration of Matrigel®, cells start to form 3D cell clusters (black arrow Figure 66F and G). No difference was observed between coatings performed at RT for 1h, 2h or 4h (data not shown). Thus we have decided to coat our plates with 0.25mg/ml Matrigel® for 1h at RT. After the coating, excess of Matrigel® was removed and cells were seeded directly.

4.1.2. Determination of the optimal seeding density

Different cell concentrations (1.6E5, 3.2E5, 4.7E5, 6.3E5, 7.9E5 and 9E5 cells/cm²) were tested to determine the optimal seeding density. Cultures were observed daily and the formation of a monolayer was monitored. All seeding concentrations tested except the highest (9E5 cells/cm²), formed monolayers within 4-15 days depending on the initial cell concentration. At the highest concentration (9E5 cells/cm2), cells did not form a clean monolayer but piled up into a multilayer (data not shown).

The quality of the monolayer was checked by labelling cells with ZO-1 in order to visualize the TJs between adjacent cells (Figure 67). TJs were visible in all conditions tested from 1.6 to 7.9E cells/cm² although for lower concentrations (1.6E5, and 3.2E5 cells/cm²) the
labeling of TJs was not completely homogenous. From these data, we selected a seeding density of 5E5 cells/cm² for the subsequent experiments since this gave homogeneous and well organized monolayers using the minimal number of primary cells.

Figure 67: Determination of the optimal seeding density visualized by the labeling of ZO-1 tight junction marker on increasing number of primary cells after one week of culture.

Initial seeding density of 1.6E5 cells/cm² (A), 3.2E5 cells/cm² (B), 4.7E5 cells/cm² (C), 6.3E5 cells/cm² (D), 7.9E5 cells/cm² (E) plated on Matrigel® coating

4.1.3. Optimisation of the size of the cluster of primary cells

Choroid plexus epithelial cells are polarized. The basolateral side is attached to a basal membrane formed by the CP stroma while the apical side extends into the lumen of the cerebral ventricles. As a consequence, for a successful monolayer, the CPe cells need to be oriented on the culture membrane and it is expected that a clumps of CP tissue will have difficulty to attach and orient correctly if they are too large (Figure 68) even in the presence of coating matrix such as Matrigel® which simulates the basal membrane. Conversely, single CP cells (Figure 68C) can attach the surface but will have difficulties to grow and form TJs with neighbouring cells. Thus the optimal size of cell clusters is critical for obtaining optimal monolayers. We determined experimentally that clusters of around 5 to 20 cells (see Figure 68D) allows efficient attachment and good growth rates with the homogeneous formation of TJs.
To obtain a preparation of small cell clusters (5-20 cells), an additional step was included in the protocol in which enzymatic digestion was performed in a 100µm nylon mesh inserted into a 6-well plate. The mesh allows passage of cell clusters containing fewer than ~20 cells which are recovered in the well. The digestion step is repeated several times in fresh wells and the released cells are pooled and centrifuged at low speed (400rpm) in order to collect preferentially the smaller cell clusters and deplete the single cells. The final procedure is described in detail in the Methods section.

Figure 68: Explanation of the optimal number of cells for primary cells attachment

Figure 69: Primary cells preparation

**Fig. A:** dissected choroid plexus from lateral ventricle before the washing step, red blood cells are still visible.

**Fig. B.** Dissected choroid plexus from the 4th ventricle. **Fig. D.** Size of the clusters after the pronase pre-digestion step. **Fig. D:** Size of the clusters after the Trypsine/DNase digestion step
Since primary CP cells are difficult to obtain large numbers from rats, we tried initially to passage the cells in order to increase the biomass available for our experiments. For this, cells were detached by enzymatic dissociation, using either StemPro® Accutase® Cell Dissociation Reagent or trypsin/EDTA solution. However, in both cases, cells reattached poorly to culture dishes and showed altered morphology under the microscope. Thus we decided not to work with passaged cells.

4.1.4. **Determination of the optimal time of culture**

Using the conditions described above, primary cells seeded at a density of 5E5 cells/cm², on transwells® coated with Matrigel® formed a monolayer as shown in Figure 70. Monolayers are complete and cells show the typical ‘cobblestone’ appearance of CPe cells.

![Figure 70: Primary choroid plexus cells form a monolayer after one week of culture](image)

4.2. **Further characterization of primary cell cultures**

4.2.1. **Presence of tight junction markers**

CPe cells express numerous well characterized TJ proteins *in vivo*. During the extraction and the preparation of the primary cells, the expression level of some proteins can vary resulting in a change of the TJ pattern and barrier properties. This pattern can also be modified by the *in vitro* culture conditions. In Figure 71, we have checked the presence of key TJ markers on primary cells by immunocytochemistry.
4.2.2. Measurement of the capacitance and resistivity

Large variations in the transepithelial electrical resistance (TEER) values for CPe cells have been described in the literature due to the use of different species (rat, mouse, cow, pig, etc.).
rabbit, …), different methodologies (in vitro or in vivo), and different types of equipment (CellZscope, or Endohm from Millipore using chopsticks). The most consistent values in vitro have been obtained by Zheng et al. at 80 Ω.cm², Southwell et al. at 100 Ω.cm² and Strazielle et al. at 178 Ω.cm².

For our experiments we have used the CellZscope and the principles of the capacitance and resistivity measurements using this instrument have been described in material and methods §5.2.4.

Continuous monitoring was performed for 72h after seeding. CPe cells isolated from the 4V (red) and the LV (blue) were analysed separately and the capacitance (Figure 72A) and resistivity (Figure 72B) were measured simultaneously.

**Figure 72: Capacitance and resistivity of primary choroid plexus cells**

Records measure with the CellZscope on a 3 days period. **Fig. A:** Capacitance measure in μF/cm² **Fig. B:** Trans Epithelial Electrical Resistance measure in Ω.cm²

For the first 24h after the seeding, the resistivity stays very low (below 10 Ω.cm²) while the capacitance starts to decrease as the cells spread across the well. A capacitance below 2μF/cm² indicates a complete monolayer which can be confirmed microscopically. By the time the monolayer is formed the cells start to express TJ proteins, which then lead to an increase in the TEER. In our experiments (Figure 72), the TEER typically starts to increase after 24h to about 95 Ω.cm² for the CP from the LV and to about 55Ω.cm² for the CP from the 4V. By 60h a plateau is reached in the TEER value indicating maximal formation of the TJs.
Figure 73: Capacitance and resistivity of primary choroid plexus cells from the lateral ventricle versus time

The Line corresponds to the average of 6 wells and the standard deviation is indicated in grey. **Fig. A:** Capacitance measure in $\mu$F/cm$^2$  **Fig. B:** Trans Epithelial Electrical Resistance measure in $\Omega$.cm$^2$  

The TEER value can be increased with some optimization as shown in the Figure 73. In this case, hydrocortisone has been added to choroid plexus primary cells and TEER values up to about $180\Omega$.cm$^2$ can be obtained. These values are consistent with comparable data shown in the literature, and cells for experimentation were taken at this time.

4.2.3. **Presence of known transporters on primary cells control by ICC and IHC**

The presence of key proteins involved in the transport by the CP cells was checked in our primary cultures. Transthyretin is a carrier protein which is normally secreted by the CP cells to transport thyroxine and retinol into the CSF and the serum. Cubilin and LRP2 are multi-ligands receptors thought to be involved in transcytosis of a diverse range of protein ligands. All three proteins are expressed in the cultured primary cells (Figure 74).
To conclude, we demonstrate that primary CPe cells express the expected signature proteins of the CP involved both in RMT and TJ formation. The presence of TJs creates a barrier which is confirmed by measurement of the electrical properties of the monolayer.

4.3. **In vitro model using Z310 CPe cell line**

For some applications the number of cells which can be obtained from the rat CP is limiting so we have also used an established rat CPe cell line, Z310. These cells were isolated by Dr. Zheng (Purdue University) and kindly provided by him for our experiments. To investigate whether Z310 cells are suitable for testing our phage derived antibodies *in vitro*, we have performed an initial characterization of these cells using immunohistochemistry, FACS and electrical properties. The presence of TJ, the capacity to form a monolayer with high resistivity, and the presence of RMT where tested (see bellow).

4.3.1. **Determination of the optimal seeding density of Z310**

As for primary cells, Z310 cultures were first tested for surface coating, seeding density and culture time in order to optimize the electrical properties of the cell monolayer. From these tests we found that $5^{E}4$ cells per 24 well insert and cells were confluent after 2 days. Coating of the plates did not affect cell adhesion and was not included in the standard protocol.
4.3.2. Measure of the resistivity of Z310

Resistivity and capacitance of the Z310 cell line was monitored in the dual chamber system for 8 days using the CellZscope and the results are presented in Figure 75. During the 5 first days, the capacitance drops from 25µF/cm² to 1µF/cm² showing that the Z310 cells form a monolayer, however the maximal TEER value obtained with the Z310 cells was about 14Ω.cm² with is much lower compared to the primary cell cultures. (§4.2.2) indicating that the barrier formed by the cell line is less tight. Thus while the Z310 cells are convenient and may yield useful indications particularly during the set up phase of the experiments in vitro, the use of primary cells is preferable for the definitive experiments to investigate transport of our candidate antibodies.

![Figure 75: TEER Measurement of Z310 cells from day2 to day 8 after seeding.](image)

**Fig. A**: Trans Epithelial Electrical Resistance measure in Ω.cm²; **Fig. B**: Capacitance measure in µF/cm².

Medium has been changed twice during the experiment.
5. *In vitro* transport experiments with reformatted candidate antibodies

From the 17 scFv-phage interesting sequences identified during the *in vivo* screening, only 11 candidates were successfully reformatted into full IgG has shown in the §3. After further characterization only 4 candidates remained (M, N, O and P), all of which derived from the *in vivo* screen using rats with long term canulation of the cisterna magna. These candidates were tested in our *in vitro* BCSFB model.

Z310 cells were grown on dual chamber plates and medium was exchanged to Optimem medium during the time of the *in vitro* transport experiment in order to improve the sensitivity of the ELISA-based quantification of the IgG candidates. TEER measurements were monitored during the duration of the exchange in order to verify the quality of the monolayer in the Optimem medium. No statistical difference between Optimem and DMEM was observed during the 1h of the experiment (data not shown) showing that the Z310 cells can stay 1h in Optimum medium without affecting the barrier properties.

The candidate antibodies and control antibodies were added at the same concentration to the bottom chamber corresponding to the blood compartment. After 1h incubation at 37°C without agitation, the medium on both side of the membrane was sampled and the concentration of IgG in both chambers was determined by ELISA as described in the methods §7.3. All samples were performed in triplicate.

![Calibration Curve using IgG M candidate as standard](image)

Figure 76A: Calibration Curve using IgG M candidate as standard
X-axis: Concentration in ng/ml; Y-axis: Elisa signal (OD$_{650}$)
<table>
<thead>
<tr>
<th>Sample</th>
<th>Replicat 1</th>
<th>Replicat 2</th>
<th>Replicat 3</th>
<th>Average</th>
<th>StDev</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Top chamber (undiluted)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG control</td>
<td>0.723</td>
<td>0.696</td>
<td>0.869</td>
<td>0.76</td>
<td>0.09</td>
<td>12%</td>
</tr>
<tr>
<td>OX-26</td>
<td>3.102</td>
<td>4.523</td>
<td>5.1</td>
<td>4.24</td>
<td>1.03</td>
<td>24%</td>
</tr>
<tr>
<td>M</td>
<td>2.031</td>
<td>2.168</td>
<td>2.04</td>
<td>2.08</td>
<td>0.08</td>
<td>4%</td>
</tr>
<tr>
<td>N</td>
<td>2.151</td>
<td>2.079</td>
<td>2.175</td>
<td>2.14</td>
<td>0.05</td>
<td>2%</td>
</tr>
<tr>
<td>O</td>
<td>1.733</td>
<td>1.876</td>
<td>2.371</td>
<td>1.99</td>
<td>0.33</td>
<td>17%</td>
</tr>
<tr>
<td>P</td>
<td>1.808</td>
<td>1.594</td>
<td>1.689</td>
<td>1.7</td>
<td>0.11</td>
<td>6%</td>
</tr>
<tr>
<td>Bottom chamber (diluted 1:500)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG control</td>
<td>0.259</td>
<td>0.246</td>
<td>0.253</td>
<td>0.25</td>
<td>0.01</td>
<td>4%</td>
</tr>
<tr>
<td>OX-26</td>
<td>0.62</td>
<td>0.599</td>
<td>0.702</td>
<td>0.64</td>
<td>0.05</td>
<td>8%</td>
</tr>
<tr>
<td>M</td>
<td>0.734</td>
<td>0.672</td>
<td>0.725</td>
<td>0.71</td>
<td>0.03</td>
<td>4%</td>
</tr>
<tr>
<td>N</td>
<td>0.782</td>
<td>0.669</td>
<td>0.937</td>
<td>0.8</td>
<td>0.13</td>
<td>16%</td>
</tr>
<tr>
<td>O</td>
<td>0.762</td>
<td>0.737</td>
<td>0.688</td>
<td>0.73</td>
<td>0.04</td>
<td>5%</td>
</tr>
<tr>
<td>P</td>
<td>Error</td>
<td>0.536</td>
<td>0.63</td>
<td>0.58</td>
<td>0.07</td>
<td>12%</td>
</tr>
</tbody>
</table>

Figure 76B: Signal of the antibodies measured on both sides of the BCSFB

<table>
<thead>
<tr>
<th>Sample</th>
<th>Replicat 1</th>
<th>Replicat 2</th>
<th>Replicat 3</th>
<th>Average</th>
<th>StDev</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Top chamber (CSF side)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG</td>
<td>1.01</td>
<td>0.97</td>
<td>1.22</td>
<td>1.07</td>
<td>0.14</td>
<td>13%</td>
</tr>
<tr>
<td>OX-26</td>
<td>4.33</td>
<td>6.30</td>
<td>7.16</td>
<td>5.93</td>
<td>1.45</td>
<td>24%</td>
</tr>
<tr>
<td>M</td>
<td>2.91</td>
<td>3.11</td>
<td>2.92</td>
<td>2.98</td>
<td>0.11</td>
<td>4%</td>
</tr>
<tr>
<td>N</td>
<td>3.08</td>
<td>2.98</td>
<td>3.12</td>
<td>3.06</td>
<td>0.07</td>
<td>2%</td>
</tr>
<tr>
<td>O</td>
<td>2.48</td>
<td>2.68</td>
<td>3.4</td>
<td>2.85</td>
<td>0.49</td>
<td>17%</td>
</tr>
<tr>
<td>P</td>
<td>2.59</td>
<td>2.27</td>
<td>2.41</td>
<td>2.42</td>
<td>0.16</td>
<td>7%</td>
</tr>
<tr>
<td>Bottom chamber (blood side)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG</td>
<td>168.75</td>
<td>159.32</td>
<td>164.39</td>
<td>164.15</td>
<td>4.72</td>
<td>3%</td>
</tr>
<tr>
<td>OX-26</td>
<td>403.96</td>
<td>387.94</td>
<td>456.13</td>
<td>416.01</td>
<td>35.66</td>
<td>9%</td>
</tr>
<tr>
<td>M</td>
<td>513.35</td>
<td>468.37</td>
<td>506.82</td>
<td>496.18</td>
<td>24.3</td>
<td>5%</td>
</tr>
<tr>
<td>N</td>
<td>548.17</td>
<td>466.19</td>
<td>660.62</td>
<td>558.33</td>
<td>97.61</td>
<td>17%</td>
</tr>
<tr>
<td>O</td>
<td>533.66</td>
<td>515.53</td>
<td>479.98</td>
<td>509.72</td>
<td>27.31</td>
<td>5%</td>
</tr>
<tr>
<td>P</td>
<td>Error</td>
<td>369.7</td>
<td>437.9</td>
<td>403.8</td>
<td>48.22</td>
<td>12%</td>
</tr>
</tbody>
</table>

Figure 76C: Concentration of antibody
CV: coefficient of variation
In Figure 76D, we present the results of transport across the \textit{in vitro} BCSFB formed by the Z310 cells. We observed significant transport of the OX-26 antibody presumably through interaction with the TfR transport pathway, and no transport of the negative IgG control. However, none of the 4 candidate antibodies M, N, O and P were transported after 1h in our \textit{in vitro} BCSFB model using the Z310 cells.
<table>
<thead>
<tr>
<th></th>
<th>Sample</th>
<th>Replicat 1</th>
<th>Replicat 2</th>
<th>Replicat 3</th>
<th>Average</th>
<th>StDev</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Top chamber (CSF side)</strong></td>
<td>IgG</td>
<td>0.95</td>
<td>1.32</td>
<td>1.79</td>
<td>1.4</td>
<td>0.419</td>
<td>31%</td>
</tr>
<tr>
<td></td>
<td>OX-26</td>
<td>13.18</td>
<td>8.96</td>
<td>8.60</td>
<td>10.2</td>
<td>2.54</td>
<td>25%</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>3.64</td>
<td>3.68</td>
<td>3.73</td>
<td>3.7</td>
<td>0.0418638</td>
<td>1%</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>3.35</td>
<td>3.59</td>
<td>3.49</td>
<td>3.5</td>
<td>0.1216885</td>
<td>4%</td>
</tr>
<tr>
<td></td>
<td>O</td>
<td>3.36</td>
<td>3.06</td>
<td>4.27</td>
<td>3.6</td>
<td>0.6307041</td>
<td>18%</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>3.16</td>
<td>1.71</td>
<td>2.89</td>
<td>2.6</td>
<td>0.770057</td>
<td>30%</td>
</tr>
<tr>
<td><strong>Bottom chamber (blood side)</strong></td>
<td>IgG</td>
<td>200.68</td>
<td>188.46</td>
<td>195.58</td>
<td>194.9</td>
<td>6.1336038</td>
<td>3%</td>
</tr>
<tr>
<td></td>
<td>OX-26</td>
<td>489.31</td>
<td>461.77</td>
<td>535.40</td>
<td>495.5</td>
<td>37.21</td>
<td>8%</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>565.10</td>
<td>562.46</td>
<td>608.18</td>
<td>578.6</td>
<td>25.668718</td>
<td>4%</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>663.83</td>
<td>553.30</td>
<td>721.96</td>
<td>646.4</td>
<td>85.677699</td>
<td>13%</td>
</tr>
<tr>
<td></td>
<td>O</td>
<td>607.20</td>
<td>653.33</td>
<td>579.05</td>
<td>613.2</td>
<td>37.503835</td>
<td>6%</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>605.45</td>
<td>550.41</td>
<td>485.17</td>
<td>547.0</td>
<td>60.214447</td>
<td>11%</td>
</tr>
</tbody>
</table>

Figure 77A: Antibody concentration in each chamber

Figure 77B: Percentage of transport (top/bottom), Percentage are calculated from the amount of IgG present in each chamber. Lower chamber volume: 1.5ml, upper chamber volume: 0.5ml

**Figure 77: in vitro transport experiment with the reformated IgG candidate on primary cells**

In Figure 77, we have repeated this experiment using primary CPe cells to generate the *in vitro* BCSFB. Antibodies were added to the lower chamber in triplicate samples as above and the incubation was allowed to continue for 1h at 37°C. Again OX-26 is actively transported across the monolayer, but disappointingly, as with the Z310 model, none of the candidate antibodies M, N, O and P showed any activity above the control IgG background.
We conclude that both *in vitro* models are able to bind and transport antibody molecules but that we were not able to validate our candidates from the in vivo screen. These results will be discussed below.
DISCUSSION

Our aim in undertaking the present project was to identify novel physiological routes into the brain with the longer term aim of exploiting any such pathway to develop a vehicle to deliver therapeutic molecules from the blood across the highly selective blood-brain barriers and into the brain tissue. This has been a major goal of numerous research programmes spanning many years, largely because the rewards in being able to develop more effective treatments for chronic neurological diseases are so great. However to date, finding molecules which could effectively deliver drugs into the brain has met with only limited success.

In our group in Merck Serono we were fortunate to have access to several key state-of-the-art technologies such as antibody phage display, as well as diverse in-house service facilities including high throughput robotics for DNA sequencing, laboratory scale protein production and downstream analyses, and a strong bioinformatics platform. In addition, the company had a wide expertise in various aspects of neurobiology and neurological procedures and a mission to develop innovative medicines. Thus we were tempted to make use of this unique opportunity to address the problem of drug delivery to the brain.

One early approach focussed on the multi-ligand cargo receptor, low-density lipoprotein receptor-related protein 2 (LRP2) or megalin, a very large multi-domain protein of over 500 kDa, present in the brain capillary endothelium. Data in the literature suggested that LRP2 is able to actively transcytose numerous ligands across the capillary cell wall such as insulin\textsuperscript{126}, Insulin-like growth factor I\textsuperscript{73}, Amyloid beta, Receptor Associated protein RAP or Leptin\textsuperscript{127} and for this reason it has been the object of several studies including the search to find synthetic peptide ligands which might provide a route into the brain. The 19 amino acid peptide Angiopep (TFFYGGRGKRNNFKTEEY) based on the so-called Kunitz domain present in certain protease inhibitors such as aprotinin, is currently being developed by Angiochem Inc as a potential brain vector for treatment of brain tumours. The main problem with approaches based on LRP2 however is the lack of organ specificity. While the receptor is abundant in brain it is also expressed in other tissues, most notably the kidney, leading to
potential problems in the clinic of rapid elimination from the circulation and possible toxicity. One way of making LRP2 a more attractive target for brain penetration would be to identify a brain specific isoform of the protein which might then allow us to to develop antibodies using a phage display-based approach, which target only the brain form of LRP2.

To investigate this possibility further, we decided to compare the mRNA coding sequences of LRP2 derived the kidney and the brain. Because of its large size, the mRNA sequence was divided into sections of approximately 2kb in length and each fragment was amplified by RT-PCR using template RNAs derived from the kidney and from the brain region known as the choroid plexus (see below). The complete mRNA sequences of LRP2 from kidney and choroid plexus were thus obtained and compared to look for any brain specific epitopes. However no differences were found between rat kidney and rat brain LRP2 mRNAs and this approach was therefore not pursued any further.

In parallel, we decided to develop an approach to screen in vivo for molecules which were able to pass from the peripheral circulation into brain. For this we adapted procedures widely used in phage display technology to establish a screening protocol for use in whole animals. We had available to us a large library of phage-encoded antibodies in a format known as single chain variable fragments (scFv). In this library, a large number of random combinations of heavy and light chain variable regions were encoded in a single polypeptide and fused to the phage protein G3p. This fusion protein is expressed on the phage surface where it is available to interact with target proteins of interest. Phage which bind the desired target can then be eluted and amplified. The power of the phage display technology is that the phenotype of the phage is physically linked to its genotype, and thus the DNA corresponding to the phage encoded antibody can be recovered and characterized further. Normally phage display screening is performed against an immobilized protein or on fixed cells in culture. For our approach we decided to perform the screen in vivo, and in this way we hoped to find antibodies which could bind to their target proteins in the natural physiological environment.

The use of phage display in vivo goes back to the late 1990's due to the pioneering work of Pasqualini and others. The early work focussed almost exclusively on the use of peptide libraries to identify phage able to bind to specific vascular beds often in an attempt
to define tumour specific targets or homing peptides for other disease states. By 2009 when we began this work, an attempt to develop a functional in vivo screen using an antibody library to investigate transcytosis was to our knowledge, completely novel.

For the reasons described below, we focussed our attention on the cerebrospinal fluid (CSF) and in essence the screen was simple. We injected the phage library into the peripheral circulation of a rat, then after a certain time interval we harvested the CSF to identify any phage present in this compartment. We reasoned that phage which were able to penetrate the CSF might do so because the encoded antibody could recognize a transporter receptor on the blood side of the barrier and be transcytosed across the capillary cell layer and into the CNS compartment. Injecting the library into the rat tail vein allowed the phage circulate in the periphery prior to coming in contact with the brain vasculature, thus non-specific binders are mostly removed during this period. The pharmacokinetics of phage in the blood has been studied previously and the half life has been estimated to be about 4h\textsuperscript{129}. From the phage harvested from the CSF we could then recover and study the properties of the associated antibody.

We chose to work with the CSF because it is a defined brain fluid which can be harvested cleanly, and its composition can be assessed relative to the composition of the blood. In this way, both sides of the blood-brain barrier can be compared directly without the need for biochemical extraction procedures. While several routes could lead into the CSF, we were especially interested in studying the choroid plexus (CP) since this tissue surrounds the brain ventricles and its principal function is to generate the CSF. Furthermore, since the turnover of the CSF is very high, (it is estimated that the CSF in man is completely replaced 3-4 times a day) we reasoned that many highly active receptors must exist on the CP which allow trafficking of molecules from the blood into the brain. Such receptors were the molecules we hoped to target in this project. An advantage of this procedure is that it makes no assumptions about the nature of the target, which potentially allowed us to identify completely unknown pathways across the blood-CSF barrier (BCSFB).

Another advantage of working with the CP is that it is a well defined anatomical structure which can be isolated by dissection and studied independently. In particular, procedures have been developed to culture the choroid plexus epithelial cells and this has allowed us
to establish a cell culture model of the BCSFB and to study the transcytosis of any candidate molecules which emerged from the *in vivo* screen, and test their ability to cross the BCSFB *in vitro*.

In attempting this approach, we were aware of several possible drawbacks which could interfere with our strategy of *in vivo* phage screening. Not least of these was the size of the phage particle itself and the question of whether such a large structure could be transcytosed *in vivo* by the cellular barriers. However, in this regard a recent paper showing that phage recognising the EGFR could be internalized by CPe cells\(^2\) suggested that this may not be an insurmountable obstacle to success.

Secondly, owing to the novelty of our *in vivo* approach, we had no reference molecules available to help establish the procedures. A search of the literature revealed several possible candidates, and the first phase of the project therefore consisted in preparing a set of potential positive control phage. We opted to construct three types of control phage which expressed (i) a scFv antibody fragment against the rat transferrin receptor (TfR), (ii) several natural ligands such as EGF and leptin which were thought to be transported across the BCSFB, and (iii) peptide ligands such as the synthetic peptide Angiopep which binds the LRP1 and LRP2 receptor and which is currently being developed commercially. The expression of all the receptors for these molecules was confirmed for the choroid plexus.

A third, and more fundamental concern for the longer term was whether the strategy of targeting the CSF would be an effective way to deliver drugs for CNS diseases. Our reservations stemmed firstly from the high turnover rate of the CSF and thus the short residence time of molecules in the brain before being returned to the blood via the sagital sinuses. This implied that any drug which could be delivered, would have only a limited time to act on its target before being eliminated. In addition, the presence of the ependyma layer confines the CSF and separates it from the brain interstitial fluid (ISF) which bathes the neurons and glia, the cells we would most likely wish to target in any disease state. Although in adults the ependyma allows relatively free exchange of molecules between the CSF and the ISF it could nevertheless retard the access of any drug molecules to the intended target cells in the brain. On the other hand, it has been shown in experimental
models of disease such as the treatment of the meningeal metastasis with antibiotics, the treatment of spasticity by the baclofen\textsuperscript{130} or for the treatment of chronic pain by opioids, that ICV injection of drug molecules offers a far more effective route than peripheral delivery which is an interesting paradigm in support of our approach, even though it may be more relevant to treatment of dysfunction within the circumventricular organs rather than regions deeper within the brain.

The major issues which we encountered in this work however were technical. Firstly isolation of CSF without traces of blood contamination proved extremely difficult. Given the high numbers of phage injected into the animal and the low frequency with which phage were expected to cross the BCSFB, contamination with even nanolitre quantities of blood resulted in overwhelming numbers of false positive phage in the harvested CSF. A sensitive assay was developed to quantify the level of blood contamination in the CSF, and several different approaches were attempted to overcome this problem as described in the results section (§2.2.3). In the end, we opted for an experimental model in which the rats were operated about two weeks prior to the experiment in order to insert a permanent canula into the cisterna magna. After healing of the surrounding tissues, this model proved to be highly successful in completely eliminating the presence of blood contamination, and in addition it allowed us to sample the CSF at several different time points following injection, thus reducing considerably the number of rats which needed to be sacrificed during the course of an experiment.

A second technical issue which confounded our early attempts to analyse the phage from the CSF was our finding referred to in the results section (§1.4.3.2) that in the second and third rounds of screening to enrich for phage which entered the CSF, the number of mutated phage increased dramatically. After testing several different explanations for the increased numbers of mutated clones, we hypothesised that the bias in the second and subsequent rounds could result from the procedure used for phage rescue. The initial library input used in round 1 was generated using hyperphage in which all G3p molecules on the phage surface are fused to the upstream scFv molecule. Since a functional G3p protein is essential for bacterial infection only those clones with an in-frame G3p protein could be propagated in bacteria thus leading to a high proportion of non-mutated scFv sequences. However, to increase stringency in the subsequent rounds we had initially
decided to rescue the phage using wild type helper phage and thus only a fraction of the G3p proteins on the phage surface are recombinant, while most are fully functional and derived from the endogenous wild type viral genome. Thus, all recovered phage are able to interact with the bacterial import machinery, and we hypothesized that this would favour selection of non-functional recombinant phage which are less toxic and more successful in terms of bacterial infection and growth rate. This would explain why a high proportion of intact clones were obtained in the round 1 while a drastic reduction in intact clones was seen in subsequent rounds.

The solution to this problem therefore was to continue to rescue all phage outputs using hyperphage, which proved to be successful and allowed us to eliminate the problem of recovering mutated phage.

Thus in the final experiments we were able to perform, in which the problems of blood contamination and phage mutation had been resolved, a number of full length phage-encoded scFvs were identified which were enriched in the CSF following multiple rounds of screening. These were considered to represent potential candidates for an antibody based vehicle to penetrate the BCSFB. The heavy and light chain variable regions of each scFv were amplified by PCR and subcloned into an appropriate vector containing the human IgG heavy and light chain framework sequences respectively. The reformatted candidate pairs of heavy and light chains were then expressed in HEK293 cells and the recombinant antibody could be purified from the culture medium. Only 4 of our positive phage clones ultimately gave good yields of the reformatted antibody while the others were lost through problems of poor expression or stability. All 4 antibodies emerged from the final screening format although disappointingly we were not able to obtain any of the antibody series which had a common heavy chain CDR3 region in combination with diverse light chains (see result section §2.3). These would have been particularly interesting to follow up on since the HC CDR3 region is generally considered to represent the major component in determining antibody specificity, and the probability that such molecules would all be enriched in the CSF should be extremely low.

In our initial programme we had intended to fuse the best candidate antibodies to a fluorescent tag and use this to follow the localisation of the antibodies after injection into
rats. Unfortunately due to lack of time we were not able to perform this series of experiments in vivo, and we decided instead to proceed directly to a fuller characterization of the recombinant antibodies in vitro.

The results of Western blot analysis in which the antibodies were probed against membrane proteins from the choroid plexus proved inconclusive. While our recombinant OX-26 antibody gave a strong signal at 110kDa, the predicted size of the rat transferrin receptor, none of our candidate antibodies showed clear evidence of interaction with a specific target protein in the CP membrane. In contrast the results obtained by ICC using rat brain slices were more promising at least for antibodies O and P which showed binding to the choroid plexus cells with an intensity comparable to that seen using the OX-26 antibody. This encouraged us to test the transport of these antibody candidates in our in vitro cell culture model of the BCSFB.

For the in vitro experiments, two models were set up in dual chamber cultures in which cells were grown on a coated synthetic membrane and both the apical (top chamber) and basolateral (lower chamber) were accessible. The first model was based on primary cells isolated from the choroid plexus epithelium (CPe), the second model made use of the CPe cell line Z310 generated in the group of Professor Zheng and kindly provided by him. Both types of culture formed tight monolayers with the expected properties as revealed using immunohistochemical, molecular biological and electrical assays as detailed in the Results section (§4). All our candidate antibodies were tested in both types of culture together with the anti-rat transferrin receptor (TfR) antibody OX-26 as a positive control, and total IgGs as a negative control. The antibodies were added to the lower chamber representing the basolateral surface which normally contacts the blood vasculature, and transport into the upper, apical chamber was measured by ELISA. Both cell types were able to transport the OX-26 positive control antibody showing that the cell based transcytosis model functionned as expected, but disappointingly the transport of none of our candidate antibodies differed significantly from the background of the negative control despite the promising results obtained using ICC. It is possible that the antibodies are able to bind to receptors on the cell monolayer but were not subsequently transcytosed across the cell, although this was not investigated.
In summary, this project was always likely to be difficult and challenging, and one with a high level of uncertainty in the final outcome. However given the ideal circumstances which existed in Merck Serono a the time to undertake this project, together with the considerable importance to medicine of a successful outcome, and the idea prevalent in the Company that 'me-too' research was not productive and that only by taking risks could we hope to achieve major progress, we considered it would be worth attempting the project described herein. In the end we were not successful in finding validated examples of phage able to transcytose into the CSF. Nevertheless the experience has been highly valuable. A new animal model for efficient sampling of the CSF was introduced into the activities of in vivo pharmacology group, and relevant bioinformatics software was improved to analyse the output of phage display data.

With hindsight, I doubt that this approach was feasible even if we had been able to resolve the different technical problems more quickly. The biological issues of transcytosing a large phage particle into the brain, together with the inherent 'stickiness' of the phage leading to an increase in the number of false positives I think inevitably would preclude a successful outcome. Tackling these issues however has been a valuable experience and in many ways it is true that we can often learn as much in failure as in success.


42. Abbott, N.J. *et al.* Specialized Neural Barriers. 2008. Ref Type: Generic


46. Pramod Dash. Blood Brain Barrier and Cerebral Metabolism. University of Texas. 2013. Ref Type: Electronic Citation


Ref Type: Thesis/Dissertation


### Appendix 1: Table of the nucleotide abbreviation according to the IUPAC

*(International Union of Pure and Applied Chemistry)*

<table>
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<th>Nucleotide code</th>
<th>Base</th>
<th>Nucleotide code</th>
<th>Base</th>
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<td>R</td>
<td>A or G</td>
</tr>
<tr>
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<td>Cytosine</td>
<td>Y</td>
<td>C or T</td>
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<tr>
<td>G</td>
<td>Guanine</td>
<td>S</td>
<td>G or C</td>
</tr>
<tr>
<td>T (or U)</td>
<td>Thymine (or Uracil)</td>
<td>W</td>
<td>A or T</td>
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<tr>
<td></td>
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<td>G or T</td>
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<td></td>
<td></td>
<td>M</td>
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<td></td>
<td>B</td>
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<td></td>
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<td>. or -</td>
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### Appendix 2: Cloning of LRP2 from kidney and choroid plexus

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<td>2</td>
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<td>032-02-bis</td>
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Appendix 3: Amino acid sequence of the scFV with non-modified linker

<table>
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<th>Signal sequence</th>
<th>Kappa light chain variable region</th>
<th>Non-modified linker present in the Hal14 vector and Hal7 library</th>
<th>Heavy chain variable region</th>
<th>6His/MYC tag</th>
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<tr>
<td>MKYLLPTAAAGLLLLAAQPAMA</td>
<td>DQLNQSPSSLASLGDITILTCHASQINIVWLSWFQQKPNAPK</td>
<td>LIVKASNLHTGVPSRFSGSGTGTGTGTTTSSFLQPEDIATYYCQQGQSYPWTFFGTTKLEIKGSASAPKLEEGFSEARYQVQLQQPAGALVRPGASMRLSCKASGYSFTTYWMNWVKQRPGQGLELIGMIHPSDEVR</td>
<td>LNQKFKDKATLTVDTSSSTAAYMLQLNSPTEDSAVYCARFGLDYWGQGTTLVSSIHNGWGRGSELDLN</td>
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Appendix 4: DNA of the GS linker used for the scFv

Linker cloned with the primers MS1240 and MS1243 (highlight in yellow); NheI restriction site is underlined

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<tr>
<th>Site</th>
<th>DNA Forward</th>
<th>DNA Reverse</th>
<th>Amino Acid</th>
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<tr>
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<td>GGGTCTGGCCGGCGGATCTGCTCGTGCGCGCGGTTGCGCGGA</td>
<td>CCCAGACCGCCGCCCTAGACCACACCAGATCGCGCGCGCGCCCGGACGGCCGA</td>
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</tr>
</tbody>
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Appendix 5: DNA sequence of rLeptin in pHal14 vector:

AATATTGAAAAGGAAAGATGATGATTTCAACATTTTCCGCTGTCGCCCTTTATTCTCCCTTTTTCGCCGCC
ATTTTGGCTCTCTGTTTTTGGCCTACCCAGAAACGCTGGTGAAGTAAAGAGATGCTGAAGATCAGTTG
GGTGCAACAGATGGTTACCATCGAATCTCAACACGGTGTAAGATGCTAGTTTCGCGCAGAGCGTACTCTACCTACCACTTTACGGATGGCATAGATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCATAGATGATGAGCAGCTGACACCACGATGCCTGTAGCAATGGCAACAACGTTGCGCAAACTATTAACTGGCG
AACTACTTACTTACTTGCTTCCGCCGACACATCAAATAAGTTAGCAGCTGGAGGAGCTAACCCTTCTCTT
GCACAAACATGGGGGATCATGTAACTCGCCTTGATCGTTGGGAACCGGAGCTGAATGAAGCCATACCA
AACGACGAAGCGTGCAACACCAGATGCTCTAGCTGAAATGGCAACACAGTGGCGTCCACTTAACTGCG
AATTTTGGCTCTTTGCTGTTTGGCGCTGCTGGCTGCTGTTTATTGCTGATAAATCTGGAGCGTGGG
TCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTATCTACGAG
CGGGGAGTCAGGCAACTATGGATGAACGAAATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAA
Appendix 6: Genotype of different bacteria used

**E. coli TG1 bacteria:**
K-12 supE thi-1 Δ(lac-proAB) Δ(mcrB-hsdSM)5, (rK-mK-) F’ [traD36 proAB+ lacIq lacZΔM15]

**XL1-Blue MRF’ bacteria:**
Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1gyrA96 relA1 lac [F’ proAB lacI0ZΔM15 Tn10 (Tef)]

**DH10B bacteria:**
F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1endA1 ara Δ139 Δ(arA, leu)7697 galU galK λ- rpsL (StrR) nupG).

Appendix 7: DNA sequence of FC5
Sequence obtained from Geneart, reference AF441486

CGAATTGGCGGAAGGCCGTCAAAGGCACTGCTTTGTCACAGCCTCCCCGGCCCATGGCAGAGGTCCAG
CTGCAGGCGTCTGGAGGAGGATTGGTGCAGGCTGGGGGCTCTCTGAGACTCTCCTGTGCAGCCTCTG
GATTCTTCTAAATACATTTACAAATGATATTGATTCTGACCATGAGACACGCCTACTATCACTGAT
ATCAGTATTACCTGGGCTTGGTGAACACCTTCTATATTTCAACACCTCGTGAAGGGCGATGAGCGT
ATTTCTAGCTGCTAGCGGTCTCCTGCTGCTGCAGGCGTCTGCCTGCAGGCGTCTGCCTGCAGGCGT
ATTATTACTGTGCAGCAGGTTCGACGTCGACTGCGACGCCACTTAGGGTGGACTACTGGGGCAAAGG
GACCCAGGGCCTCAGGATGTATCATGACGTCATGACGTCATGACGTCATGACGTCATGACGTCATGACG
GCAGGCGATGAGCGCAGGCGATGAGCGCAGGCGATGAGCGCAGGCGATGAGCGCAGGCGATGAGCGC
TTCCGCTCAGCTGC

Appendix 8: Amino acid sequence of FC5
Appendix 9: DNA sequence from FC44
Sequence obtained from Geneart, reference AF441487)

CGAATTGGCGGAAGGCCGTCAAGGCCACGTGCTTTTGCACCAGCTCCGGCCATGCAGAGGTCAG
CTGCAGGCGTCTGGGGGAGGATTGGTGCAGGCTGGGGGCTCTCTGAGACTCTCCTGTTCAGCCTCTG
TACGAGCTCAGATATATGCACATGCCATGGGCTGGTTCCGCCAGGCTCCAGGGAAGGAGCGTGAGTTTGT
AGCAGGTATATACCGAAGTGGTGATGGATCGTACAAAGTATGCAGACTTCGTGAAGGGCCGATTCCATC
TCTAGAGCAACACCCGAGAAACTGGTCTGACATCCTGGAACATGGTGTACCTGCAAATGAATAGCCTGAA
ACCTGAGGACCCAGGTCACCGTCTCCTCTCAGCGGCCGCACATGGTACCTGGAGCACAAGACTGGCC
TCATGGGCTTCCCGCTCACTGC

Appendix 10: Amino acid sequence of FC44

EVQLQASGGGLVQAGSRLSLCAASFKITHYTMGWFRQAPGEREFVSRTWGGDNTFYSNSVKGRFTISRDN
AKNTVYLQMSLKPEDTADYYCAAGSTSTATPLRVDYWKGKTQVT

Appendix 11: Sequenced DNA from the Light Chain Forward of OX-26
Raw data SPL02636B05

NNNNNNNNNTGNATGACNTCCAATGGGTTCCCTCTCCNCAGGTTGCACCACCTCCAGGTCCCA
AGTTTAAACGGATCCTTAGCAATTGCCGGCCACCATGGGACACCCCTGCTGCTGGGCTGCT
GCTGGCTGGGTTGCCCAGCCGATGACAGCCGATTTGAGATACCTCGATGTCAGCTGGGCT
TCTCGGACATCTCTGAGCTGACCTGTGGTGGCTGATCTCCATCCAGTCTGTGTCAG
TCACCAGGAAATGCCTCTAAACTGTGTAATTGCTTTACCTCGACATCCAGGAGGCT
CCCATCAAGATTTTAGCTGTGGGCAAAGCTGGTTTACTCGATGACAGCCGATCT

183
Appendix 12: Sequenced DNA from the Light Chain Reverse of OX-26
Raw data: SPL02636C01

Appendix 13: Sequenced DNA from Heavy Chain Forward of OX-26
Raw data: SPL02636C105
Appendix 14: Sequenced DNA from the Heavy Chain Reverse of OX-26

Raw data: SPL02636D06

Appendix 15: Restriction enzymes used

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<th>Enzyme</th>
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<td>EcoRI</td>
<td>5'GAATTC 3'CTTAAG</td>
<td>5'---G AATTC---3' 3'---CTTAAG G---5'</td>
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<tr>
<td>BamHI</td>
<td>5'GGATCC 3'CCTAGG</td>
<td>5'---G GATCC---3' 3'---CCTAGG G---5'</td>
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<tr>
<td>HindIII</td>
<td>5'AAGCTT</td>
<td>5'---A AGCTT---3'</td>
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</table>
Appendix 16: mRNA sequence of mouse LRP2

>gi|124487371|ref|NM_001081088.1| Mus musculus low density lipoprotein receptor-related protein 2 (Lrp2), mRNA

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<tr>
<th>Enzyme</th>
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<th>3’TTCA A—5’</th>
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<td>5’GCGGCCGC</td>
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<tr>
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<td>3’CCGCCGGCG</td>
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<tr>
<td>XbaI</td>
<td>5’TCTAGA</td>
<td>5’TCTAGA</td>
</tr>
<tr>
<td></td>
<td>3’AGATCT</td>
<td>3’AGATCT</td>
</tr>
<tr>
<td>XhoI</td>
<td>5’CTCGAG</td>
<td>5’CTCGAG</td>
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<tr>
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<td>3’GAGCTC</td>
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<tr>
<td>BsrGI</td>
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<td></td>
<td>3’ACATGT</td>
<td>3’ACATGT</td>
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<tr>
<td>NotI</td>
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<td>3’CAGCTG</td>
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</table>

Note: The sequences are given for both strands as they are complementary.
Appendix 17: Amino acid sequence of LRP2

>sp|P98164|LRP2_HUMAN Low-density lipoprotein receptor-related protein 2 OS=Homo sapiens GN=LRP2 PE=1 SV=3

MDRGPAAVACTLLLALVACLAPGSGEQCSHHFRCGSGHCIPADWRCDGTKDCSDDEADEI
GCAVTCQQYFKCQSEQCIPNISWCVQDQDCDDGSDERQDCSQTSCSHQITCSNGQC
IPSEYRCVHRDPCADENDCQYPTCEQLTCDNGACYNTSQKCDXKVDRCRDSDEINC
EICLHNFEFCNGEICPHRYCDHNDQDSDEHAHNYPTCQGGYQFTCPSCGRSRYCNQW
GDCEGDDGDNCDEGCGEHPDHCKSVPRESWCPESREPICYSVCDGILDCGDREDENDN
TSNGYKCMSTLACNLQCYCHETYGACCPFPGYIINHNSDRTCVEFDCQIQWIGDCQ
KCESPRGHLCHCEEGYLERCQYCKANDSFGEASYIFNSGDRDLDIHIHGRSRIFLVES
QNVRGNYAVQFHLYLHQRVFWDTVQNKVSVDINGLNIQVELVNSVETPENLAVDWNKNK
ILYVETKNVRIDMNDSYVRLTILENHLHRPIAVDPTVGLYLFSDNWSLSEGFKLER
AFMDGSNRKDLRDVFCHVGYTDVIIKSMYRVDSFDYETEVTYDIQRKTVWVHGSL
1PHFVGSLFXQFQUTWTXDKMVALKFBKFTETNQYYQASLRPGYTVYHSLQRAYAT
NPCDKNCGNCVQHLSNDGGLFRCXCTGFQFLDTERICAYQVNLFLSSQVAIR
GIFPLTSLQEDMVFVSNPSFGFYIGDAYEDFQQTFDSTIFSDFSMKSHIFQKIDTGREILAA
NRVENSVLAFDWISKYNLTDHSYKISVYMRLKDRTRTVQYNNPRSVVVFAGYL
FTDTNFWRPAKIMRAWSDGHLLPVTILLGWNLAGIAWDASRLYWVDAYFDKIEHSTFD
GLDRRRLGHIEMTHFFGLAIHEULFTTDWRLGAIIRVKADGEGMTTISGIAIYIHL
KSYDVNQTPSNAQCPNQPHNGDSHCFCFPFVNPQRFVQCCYGMRALSNHTCQDPTNE
PTEQCCGFSLSCFCNGVZNYLDCGVDCHDNSDEQLCGTLNNTCSSSAFTCQGHECI
PAHWRCDKNRCDVQSDHENCPTAPASCLTDYTCNDHCQISKWNCVDTCDCGDGSDE
KNCNSETETCPQSNCPHRICDLSVFCGDKDCVSGDSEQGVLNCNTASQFKASGDKC
IGVTNRCDQCVFDSNSDNEAGCFRPPGCMCSEDCQEFDCICIPMNFWCHGDHDCLYG
DEHNAVCYKPTFPCSYHCDNGCNICHRAWLCRLRDNCDGMSDEKDCPTQFPPCSPWQQC
GHNINCVNLNSVCDGFIDCPGNTDESLCNAGCGHETEQVEFPGAACLFLGFL
LANDSCTICEDCILQSHCYNMGKRSFCSDCTGYLMSGDRTCKVTASELILLV
ASQNKIDAVSNTSVQHNIYSLVENGTSQVFDSIFISGRFSDATQKTSWAFQGNTD
RVRFDSIIIETIAIDVGRNLNYTDYATEVIEXSKDGSHRTVLNISKLNTPNGLAL
PRMNEHLLFSWDSWHHPRIERASMDSGMSRTVIVQDKIFPCGLTIIDYPNRLLYLMYSYLD
YMDFCNDYNGHRQRVIAASLDRHIHPYATLTFESVYWTDRATRVRMVANKWHGNQSVVM
YNQVLQWPHVQSPKNVSACFPCRSHCLLSSQGPHYSCVCFSWLSLFDNLNC
LRDQDPQFLVTIRQIIIFGISNLPCVEKSNAMDIQAIGQNGLVEFDDAEQYIYWENFGE
IHVRKDGTNRTFVASMVPSMNLSMALWIRNSLYSTNPRQSIELTDEHGIRYKTL
IANDTALGVPFPFTVIGTDAPRKLGYSDQTDGVPFAKASINAMDGTVSTKLFLENGLE
LECVLTLDIEQKLYWAVTGRIVERGNDTDLRMLVHQLSHPGWIAVHSDFLFYTEDEQ
EVIERDVKTAGANKTVLDRDNPNLRQLQYHVHRNAEESNSCNMNACQICLPFVPGGL
FSACACGTFKIPNDRSCSNCPSYEFVSMIALRFSLELDHESTMVFGAGNRNALHY
DVSVDSTSFYWWCDFSVSANHAIKRPDGSSLSNMVTHIGENGVRGLAVDVWAGNLY
FTNAFVSETLIEVRLNTTYVRLKVLTDMDPFIHVIKDNRYLFWDYAGQFKPERSFL
DCTNRVTLVESEVQFTVRGLAVRDSGYYWVLDSDIARIRINGENSEVYGRSYFTP
YGITYFVSENSIIWDRNLKFLKIQASKEPEPTEPPTVIFIRDINVNLNIRGDVTIFDKQVFPRSPAE
VNPNPLCENNGLSHCPLALGPHLPHTKDCAFATGLQDSKGNAISTENFLIFALNSLRS
LHLDENPENSSPFQTINTERVMNVSLDVSIVDRYFPQNLASGVGQRYSATLSGOTHFTP
IAGSRTAGDIADFWITRRIYISDLYNQIMNSAEDGNSRTVIAFVPRAFALDPCQY
### Appendix 18: Nucleotidic Sequence of the primers

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<th>Primer ID</th>
<th>Description, Direction</th>
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<td>MS917</td>
<td>RT-primer-2</td>
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<td>Bottom strand PCR primer (NotI)</td>
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<td>MS920</td>
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<td>MS923</td>
<td>Bottom strand PCR primer (NotI)</td>
<td>AACCGCGGCCGC CTATCCACCAATGATAGAC</td>
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<td>MS924</td>
<td>Top strand PCR primer (NotI)</td>
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<td>Bottom strand PCR primer (NotI)</td>
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</table>

RT-PCR and PCR primers of rat LRP2
<p>| MS1084 | RT primer for ligand binding domain 3 | CCACAACATTGCCCAATCCTTGCC |
| MS1085 | Fwd PCR primer for ligand binding domain 3, &quot;Fragment B&quot; | GTGGACACGGGGACCCTGTAATC |
| MS1086 | Reverse Primer for ligand binding domain 3 to BsrGI site, &quot;Fragment B&quot; | GTTATGGTATCGTACAGTGGTG |
| MS1087 | Fwd PCR Primer - NsiI, &quot;Fragment D&quot; | GATGTGCTGATCAGCAATGCATCC |
| MS1088 | Rev PCR - XhoI, &quot;Fragment D&quot; | GGATGCCTCTCGAGAGCCCAACCTC |
| MS1089 | Fwd PCR primer - XhoI | GAGGTGGGCTCTCGAGAGGCATCC |
| MS1090 | Signal Seq Fwd PCR Primer (XbaI), &quot;Fragment A&quot; | GCTCTAGAATGGAGCGCAGCCAGCGCGGGCGCTGATGCTGTTGCTGGGATCGCTG |
| MS1091 | Signal Seq Rev PCR Primer (NotI), &quot;Fragment A&quot; | GGTCCTCGTGTCACGCTACTGACTGCTCCAGGCAGCCAGCGATCGCCAGCAAC |
| MS1110 | (Fwd nested primer for MS926) (NotI) (use with MS1111) | AACCAGCGCCGCCTCCATCAATTCCGGGT |
| MS1111 | (Rev nested primer for MS927) (NotI) (use with MS1110) | AACCAGCGCCGCATCGCATGCCAAGCAGCCTT |
| MS1112 | (Fwd nested primer for MS1087 (NsiI) (use with MS1113) | CAGCAATGCATCCCTCTCGATGGGTCTTG |
| MS1113 | (Rev nested primer for MS1088 (XhoI) (use with MS1112) | TGCCCTCGAGAGCCCAACCTC |
| MS1114 | (Fwd nested primer for MS1085) (use with | CACGGGACCCGCTGTAATCAACTCCAGTTCAC |</p>
<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Description</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>MS1115</td>
<td>(Rev nested primer for MS1086) (BsrGI) (use with MS1114)</td>
<td>GGTATCTGTACAGTTGTGGTCACAGCG</td>
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<tr>
<td>MS1116</td>
<td>(Replaces MS1091) (use with MS1090)</td>
<td>CAGCGGGGTCCCCGTGCACGCTACTGACCTCCAGGCCAGCGATCGCCAGCAAC</td>
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<td>MS1117</td>
<td>5' Fwd primer (SalI) (Use with MS1115)</td>
<td>AACCGTCGACGCCACCATTGAGCGCCGGGGCCGCAAGCG</td>
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<td>MS916</td>
<td>RT-primer for cDNA-1</td>
<td>GTTCCCTAGAGAGGCTGGCGAG</td>
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<td>MS1084</td>
<td>RT primer for cDNA-2</td>
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<td>MS1090</td>
<td>Fragment A Fwd PCR Primer (XbaI)</td>
<td>GCTCTAGAATGGAGCGGCCGCAGCGGCGGCGTGGATGTTGCTGGCGATCGCTG</td>
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<td>MS1091</td>
<td>Fragment A Rev PCR Primer (NotI)</td>
<td>GGTCAGATGAGCGCCGCTGGCAGCGGAGCAAGCAGCGATCGCCAGCAAC</td>
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<td>MS1085</td>
<td>Fragment B Fwd PCR primer</td>
<td>GTGGACACGGGGGACCGGCTG</td>
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<td>MS1086</td>
<td>Fragment B Reverse Primer (BsrGI) and to assemble A and B</td>
<td>GATTATGTATCGTACAGGGTGG</td>
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<tr>
<td>MS1117</td>
<td>Fwd PCR primer (SalI) to assemble A and B</td>
<td>AACCGTCGACGCCACCATTGAGCGCCGGGGCCGCAAGCG</td>
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<td>MS926</td>
<td>Fragment C Fwd PCR primer (NotI)</td>
<td>AACCGCCGGCGGC GATGAGCGAGGAGCACCTG</td>
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<td>Fragment C Rev PCR primer (NotI)</td>
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<td>Fragment D Fwd PCR primer - Nsi</td>
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<td>MS1121</td>
<td>Fragment D Rev PCR primer (NotI)</td>
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<td>Use of the primers</td>
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<td>Cloning of OX-26 VL and VH into pTT5 vector</td>
<td>MS1215</td>
<td>VkL top strand (SalI): , AACC. Irrelevant at the beginning to help the enzyme to bind</td>
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<td>MS1216</td>
<td>VkL bottom strand (BsiWI): , AACC. Irrelevant at the beginning to help the enzyme to bind</td>
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<td>MS1217</td>
<td>VH top strand (BamHI): , AACC. Irrelevant at the beginning to help the enzyme to bind</td>
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<td>MS1218</td>
<td>VH bottom strand (NheI): , AACC. Irrelevant at the beginning to help the enzyme to bind</td>
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<td>Cloning of OX-26 into pHal14 vector in VL-VH orientations</td>
<td>MS1220</td>
<td>VL-fwd (NcoI):</td>
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<td>MS1221</td>
<td>VL-rev (HindIII):</td>
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<td>MS1222</td>
<td>VH-fwd (MluI):</td>
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<td>MS1223</td>
<td>VH-rev (NotI):</td>
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<tr>
<td>Cloning of OX-26 into pHal14 vector in VH-VL orientations</td>
<td>MS1224</td>
<td>VH-fwd (NcoI):</td>
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<td>MS1225</td>
<td>VH-rev (HindIII):</td>
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<td></td>
<td>MS1226</td>
<td>VL-fwd (MluI):</td>
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<td></td>
<td>MS1227</td>
<td>VL-rev (NotI):</td>
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<tr>
<td>Cloning of scFV OX-26 into pHAL</td>
<td>MS1240: Bottom strand</td>
<td>LINKER-VLrev (use GCCGCGCCCTAGCACCACCAGATCCGCCGACCCTTTAGATTCCAGCTTGGTG</td>
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<td>with a GS linker, OX26-VL-Gs linker-VH</td>
<td>with MS1220):</td>
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<td>MS1241:</td>
<td>Top strand linker-VHfwd (use with MS1223):</td>
<td>GGTGGTGTAGCCGGCGGGGCGCTGGCGGGACAGGTCTCACTCCAACAGC</td>
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<td>Cloning of scFV OX-26 into pHAL with a GS linker, OX26-VH-Gs linker-VL</td>
<td>Bottom strand Linker-VHrev (use with MS1224):</td>
<td>GCGCCGCTAGCACCAGATCCGCCAGACCCGGAGGAGACTGTGAGAGTGG</td>
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<td>MS1242:</td>
<td>Top strand linker-VLfwd (use with MS1227):</td>
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<td>PCR out the scFv fragment from HAL7 vector, - VL chains HAL7 (to clone into pTT_CL_20968)</td>
<td>VL1BssHII_FORpTT5</td>
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<td>VL4BssHII_FORpTT5</td>
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<td>VL5BssHII_FORpTT5</td>
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<td>IGLJ1_Fse</td>
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<td>IGLJ4_Fse</td>
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<td>IGLJ2/3/5_Fse</td>
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<td>IGLJ6_Fse</td>
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<td>IGLJ7_Fse</td>
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<td>PCR out the scFv fragment from HAL7 vector, - VK chains HAL8 (to clone into pTT_CK_20967)</td>
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<td>IGLKJ1/J4_Not</td>
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<td>IGLKJ2_Not</td>
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<td>IGLKJ3_Not</td>
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<td>IGLKJ5_Not</td>
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<td>PCR out the scFv fragment from HAL7 vector, - VH chains HAL7 &amp; UnilacFOR pool</td>
<td>UniLacFOR pool:</td>
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<td>HJ1Sal_REVpTT5</td>
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<td>HJ2Sal_REVpTT5</td>
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<td>HJ3Sal_REVpTT5</td>
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<td><strong>HAL8</strong> (to clone into pTT_CH1_20966)</td>
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<td><strong>PCR primer, Forward</strong> + <strong>BamHI</strong></td>
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<td>MS931</td>
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<td><strong>Sequencing of BSK vector</strong></td>
<td><strong>T3</strong></td>
<td><strong>T7</strong></td>
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<td><strong>Sequencing of pHal14 vector</strong></td>
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<td><strong>pHALG3 REV</strong></td>
<td><strong>Reverse</strong></td>
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