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TITLE:

A Human Blood-Brain Interface Model to Study Barrier Crossings by Pathogens or Medicines and Their Interactions with the Brain

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KEYWORDS:

BBB-Minibrain, in cellulo model, Human endothelial cells hCMEC/D3, Ntera/Cl2D.1, Human neuron, Human astrocyte, Human microglial cells CHME/Cl5

SUMMARY:

Here we present a protocol describing the setting of an in cellulo BBB (Blood brain barrier)-Minibrain polyester porous membrane culture insert system in order to evaluate the transport of biomolecules or infectious agents across a human BBB and their physiological impact on the neighboring brain cells.

ABSTRACT:

The early screening of nervous system medicines on a pertinent and reliable in cellulo BBB model for their penetration and their interaction with the barrier and the brain parenchyma is still an unmet need. To fill this gap, we designed a 2D in cellulo model, the BBB-Minibrain, by combining a polyester porous membrane culture insert human BBB model with a Minibrain formed by a tri-culture of human brain cells (neurons, astrocytes and microglial cells). The BBB-Minibrain allowed us to test the transport of a neuroprotective drug candidate (e.g., Neurovita), through the BBB, to determine the specific targeting of this molecule to neurons and to show that the

neuroprotective property of the drug was preserved after the drug had crossed the BBB. We have also demonstrated that BBB-Minibrain constitutes an interesting model to detect the passage of virus particles across the endothelial cells barrier and to monitor the infection of the Minibrain by neuroinvasive virus particles. The BBB-Minibrain is a reliable system, easy to handle for researcher trained in cell culture technology and predictive of the brain cells phenotypes after treatment or insult. The interest of such in cellulo testing would be twofold: introducing derisking steps early in the drug development on the one hand and reducing the use of animal testing on the other hand.

INTRODUCTION:

The brain is separated from the systemic circulation by a non-permeable structure that restricts exchanges between the brain parenchyma and the blood, called the blood-brain barrier (BBB). Mostly composed of cerebral endothelial cells, the BBB dynamically interacts with astrocytes, perivascular microglia and neurons of the neighboring brain parenchyma. The three major functions of the BBB are the creation and maintenance of ionic homeostasis for neuronal functions, supply of the brain with nutrients, and protection from toxic injuries or entry of pathogens[1,2], which contribute to the maintenance of brain homeostasis and its functions[3]. This barrier is so efficient that only few drugs can cross the BBB[4,5]. At present, the available methods to predict whether a molecule will pass the BBB and diffuse into the brain consist of ex vivo studies on autopsy material, image tracking in the brain of human volunteers by MRI (magnetic resonance imaging) or PET (positon emission tomography) or pharmacodynamics and pharmacokinetic preclinical studies in animals[6-8]. These techniques and models have some limitations, such as the limited resolution of PET and the low sensitivity of MRI[6,8], the difficulty to quantify molecules (i.e., antibody based molecules for example) that poorly penetrate the brain[7], and for the preclinical studies their high cost and resort of animal testing.

The last point is important because, according to the 3R's rules, (replacement, reduction and refinement of animal testing) the regulatory administrations have asked that the researchers urgently develop scientifically accurate alternative to animal experimentation[9-15].

Over the last decades, several in vitro models of BBB have been proposed[16-18] by cultivating on filter membrane inserts endothelial cells from different species such as mouse, rat, bovine and pig. As far as the human species is concerned, the scarce and difficult availability of primary cells prompted the researchers to develop human models based on immortalized brain endothelial cells or human-derived stem cells[19-21]. These barriers are proper in vitro surrogates of BBB provided that they express endothelial cell markers, tight junction markers, efflux transporters, solute carriers, receptors, and respond to the endothelial stimuli [20]. A few BBB models using filter membrane inserts coated with endothelial cells and other cell types (i.e., astrocytes, neurons or pericytes[22-24]) were assayed. The goal of these co-cultures was to increase the BBB physical characteristics by taking advantage of the secretion of soluble factors by astrocytes/neurons or pericytes.

Nevertheless, none of these models includes brain parenchyma to study and predict the fate of a drug candidate once it has passed the barrier. Therefore, our goal was to build an in cellulo

blood/brain interface, the BBB-Minibrain, by combining a BBB model and a culture of mixed brain cells into a single kit. The BBB-Minibrain uses a culture system consisting of a porous filter inserted in a well of a multiwell cell culture plate. The filter is coated with hCMEC/D3 cells, a human brain endothelial cell line that has been proved highly reliable for BBB drug testing[25-27], to form the BBB. The Minibrain, which is a co-differentiated culture of human neurons and astrocytes derived from the NTera/Cl2.D1 cell line[28,29] mixed together with the human microglial cell line CHME/Cl5[30] in ratio corresponding to the microglia vs. neuron-astrocytes ratios of the brain[31], is cultivated in the bottom of the plate well.

Besides studying passage of drugs across the BBB and their fate in the parenchyma, the blood-brain interface in cellulo model could be a powerful tool to address the entry of pathogens into the brain (neuroinvasiveness), the dispersion into the brain (neurotropism) and the toxicity (neurovirulence) they can exert on brain parenchyma cells. Neurovirulence and neuroinvasiveness studies would benefit from the development of an efficient in cellulo model and be advantageous to replace animal models. Using the BBB-Minibrain kit[32], we demonstrated the neuroinvasive phenotype of rare viral mutants that accumulated in the French Neurotropic virus strain of Yellow Fever Virus (i.e., FNV-YFV[33,34]) used to prepare a discontinued live YFV vaccine and the passage of a neuroregenerative and neuroprotective biomolecule called Neurovita (referred as NV henceforth in the manuscript)[35]. Because NV neither naturally crosses the cell membrane nor the BBB, NV was fused with the variable part (VHH) of a single chain antibody of Llama that crosses the biological membranes including the BBB and functions as a cell penetrating molecule (CPM)[36]. The CPM property of VHH seems to depend upon the isoelectric point and the length of the VHH[37].

This in cellulo test should make it possible to sort the molecules that could potentially cross the BBB before carrying out pharmacokinetic and pharmacodynamics analysis in animals, and ideally in the same time to be able to predict their behavior in the nervous parenchyma. This system is biologically relevant and easy to set up and handle by professionals well trained in cell culture[26,29,30,38]. The interest of such in cellulo testing would be two-fold: reducing the costs of preclinical tests on the one hand and reducing the use of animal testing on the other hand.

PROTOCOL:

1. Cell culture work of Ntera/CL2.D1 to prepare a co-culture of post-mitotic hNeurons and hAstrocytes (NT2-N/A)

NOTE: This is the component of the Minibrain (**Figure 1**).

1.1 Culturing the Ntera/Cl2.D1

1.1.1 Remove a vial of frozen cells from liquid nitrogen tank. Keep on ice.

1.1.2 Thaw the cells rapidly in a 37 °C water bath.

1.1.3 Transfer the cells in a 15 mL tube containing 10 mL of complete DMEM F12 medium (Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 medium) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 IU penicillin and 100 µg streptomycin (i.e., complete DMEM F12 medium).

1.1.4 Centrifuge at 200 x *g* for 5 min at room temperature (RT). Dissociate the pellet in 2 mL of complete DMEM F12 medium.

1.1.5 Transfer in a T75 tissue culture flask in polystyrene with specific treatment for sensitive adherent cells (T75Cell$^+$) containing 13 mL of complete DMEM F12 medium.

1.1.6 Culture cells in an incubator maintained at 37 °C, 5% CO_2 and 95% humidity until 90% confluence (i.e., around 5 days). Change medium every 2-3 days.

1.2 Subculturing the Ntera/Cl2.D1 and amplification

1.2.1 Remove the medium from the flask.

1.2.2 Rinse the cell monolayer with 10 mL of phosphate buffer saline (PBS) supplemented with Ca^{2+} and Mg^{2+}.

1.2.3 Rinse the cell monolayer with 10 mL of EDTA solution (kept at RT).

1.2.4 Add 3 mL of trypsin-EDTA solution (0.05% trypsin, 0.02% EDTA) and incubate 2 min at RT.

1.2.5 Shake the flask and make sure the cells are detached from the plastic surface.

1.2.6 Add 10 mL of complete DMEM F12 medium to inactivate the trypsin, transfer in a 15 mL tube and centrifuge for 5 min at 200 x *g* at RT.

1.2.7 Dissociate the pellet with 10 mL of complete medium and use 1 mL to inoculate each new flask containing 14 mL of complete DMEM F12 medium.

NOTE: Thirty T75 Cell$^+$ flasks are required for each differentiation.

1.2.8 Incubate the flasks at 37 °C, 5% CO_2 and 95% humidity until 90% confluence.

1.3 Differentiation of NTera/Cl2.D1 in human neuron-astrocyte co-culture

NOTE: This is the main component of the Minibrain.

1.3.1 At Day 0, dissociate the cells with trypsin-EDTA as described in step 1.2 and dissociate the cell pellet of each flask with 2 mL of complete DMEM F12 medium.

1.3.2 Add 1 mL of the cell suspension (corresponding to 5 x 10^6 cells) in 60 plastic Petri dishes of 85 mm diameter containing 11 mL of complete DMEM F12 medium.

NOTE: The cells will not attach to the plastic and will aggregate to form pseudo neurospheres (see photograph in the lower panel of **Figure 1**). Incubate the 60 Petri dishes at 37 °C, 5% CO_2 and 95% humidity for one day.

1.3.3 At Day 1, add 1 mL of complete DMEM F12 medium supplemented with 130 µM of all-Trans Retinoic Acid (ATRA) per Petri dish (final concentration ATRA 10 µM) and return the dishes at 37 °C, 5% CO_2 and 95% humidity for 24 h.

1.3.4 At Day 2, transfer very carefully the medium containing cell spheres of each Petri Dish in a 50 mL tube and centrifuge for 10 min at 50 x *g* and RT. Dissociate carefully the loose pellet with 13 mL of complete DMEM F12 medium supplemented with 10 µM ATRA and add the 13 mL in a new 85 mm diameter Petri dish.

NOTE: Over the different passages, it is extremely important to maintain the spheres density as high as the density obtained at Day 2 (i.e., around a density of 70%). Therefore, if the sphere density is lowering, reduce the total number of Petri dishes according to the number of cells.

1.3.5 Repeat the procedure above (step 1.3.4) at days 4, 6 and 8.

1.3.6 At Day 10, repeat the procedure above but add the spheres to the T75 Cell$^+$ flasks instead of Petri dishes. Add the cells from one Petri dish to one T75 Cell$^+$ flask.

1.3.7 At Days 11, 13, 15 and 17, change the used medium with 14 mL of complete DMEM F12 supplemented with 10 µM ATRA.

1.3.8 At Day 19, change medium with 14 mL of complete DMEM F12 medium without ATRA per flask.

1.3.9 At Day 20, dissociate gently the cells with trypsin/EDTA as follows.

1.3.9.1. For each T75 Cell$^+$ flask, rinse the cells with 10 mL of PBS supplemented with Ca^{2+} and Mg^{2+}; incubate the cells with 4 mL of EDTA for 5 min at RT.

1.3.9.2. Remove carefully the EDTA and add 2 mL of trypsin-EDTA and incubate 7 min at RT. Shake very gently the flask and add carefully 8 mL of complete DMEM F12 medium.

1.3.9.3. Centrifuge 10 for min at 160 x *g* and RT. Dissociate the cell pellet in 13 mL of complete DMEM F12 medium and transfer in a T75 Cell$^+$ flask.

NOTE: The very gentle dissociation with trypsin-EDTA will allow recovering the ATRA differentiated cells from the original teratocarcinoma cells, which are strongly attached to the

plastic ware.

1.3.10 At Day 21, remove the medium and the dead cells. Add 14 mL of complete DMEM F12 medium supplemented with 5% FBS, 2 mM glutamine, 100 IU penicillin, 100 µg streptomycin and 0.5 µM of AraC (Cytosine β-D-arabinofuranoside) (complete 5% FBS DMEM F12-AraC).

1.3.11 At Days 23, 25 and 28, replace used medium with 14 mL of 5% FBS DMEM F12-AraC.

1.3.12 At Days 29 up to 49 (every two days), replace used medium with 14 mL of complete DMEM F12 medium supplemented with 5% fetal bovine serum, 2 mM glutamine, 100 IU penicillin, 100 µg streptomycin, 5 µM of FudR (5-fluoro-2'deoxyuridine) and 10 µM Urd (Uridine) (complete 5% FBS DMEM F12-FudR-Urd).

1.3.13 At Day 50, replace used medium with 14 mL of complete DMEM F12 medium supplemented with 5% FBS, 2 mM glutamine, 100 IU penicillin, 100 µg streptomycin and 10 µM Urd (complete 5% FBS DMEM F12-Urd).

1.3.14 At Days 51 up to 95 (twice a week), replace used medium with 14 mL of complete 5% FBS DMEM F12-Urd.

NOTE: Cells can be used for experiments from day 51 but not later than day 95. The use of serial treatments with mitosis inhibitors allows recovering pure population of co-culture of post-mitotic hNeuron and hAstrocytes (NT2-N/A).

1.3.15 To seed the cells, proceed with gentle trypsinization as described for Day 20.

2. Cell culture work of human microglial cells CHME/Cl5

NOTE: This is the microglial component of the Minibrain **(Figure 1).**

2.1 Culturing the human microglial cells CHME/Cl5

2.1.1 Remove a vial of frozen cells from the liquid nitrogen tank. Keep on ice.

2.1.2 Thaw rapidly in a 37 °C water bath.

2.1.3 Transfer the cells in a 15 mL tube containing 10 mL of complete DMEM F12 medium supplemented with 5% fetal bovine serum, 2 mM glutamine, 100 IU penicillin and 100 µg streptomycin (i.e., complete 5% FBS DMEM F12 medium).

2.1.4 Centrifuge at 200 x *g* for 5 min at RT. Dissociate the pellet with 2 mL of complete 5% FBS DMEM F12 medium.

2.1.5 Transfer in a T75 Cell^{+} flask containing 13 mL of complete 5% FBS DMEM F12 medium.

2.1.6 Culture cells at 37 °C, 5% CO_2 and 95% humidity until 90% confluence. Change medium every 2-3 days.

2.2. Subculturing the human microglial cells CHME/Cl5

2.2.1 Remove medium from the flask.

2.2.2 Rinse the cell monolayer with 10 mL of PBS supplemented with Ca^{2+} and Mg^{2+}.

2.2.3 Rinse the cell monolayer with 10 mL of EDTA solution (kept at RT).

2.2.4 Add 3 mL of trypsin-EDTA solution and incubate 2 min at RT.

2.2.5 Shake the flask and make sure the cells are detached from the plastic surface.

2.2.6 Add 10 mL of complete 5% FBS DMEM F12 medium to inactivate the trypsin, transfer in a 15 mL tube and centrifuge for 5 min at 200 x *g* and RT.

2.2.7 Dissociate the pellet with 10 mL of complete medium and use 1 mL to inoculate each new flask containing 14 mL of complete 5% FBS DMEM F12 medium.

2.2.8 Incubate the flasks at 37 °C, 5% CO_2 and 95% humidity until 90% confluence.

3. Culture work with the hCMEC/D3 to coat porous inserts and prepare BBB

3.1 Culturing the human endothelial cells hCMEC/D3 (**Figure 2**)

3.1.1 Dilute the type I rat Collagen to 1:30 with pure sterile water (cell culture grade).

3.1.2 Transfer 10 mL into a T75 Cell$^+$ flask and incubate 2 h in a 37 °C, 5% CO_2 and 95% humidity incubator.

3.1.3 Remove the collagen solution and replace it with 15 mL of endothelial cell medium supplemented with 10 mM of HEPES (referred as complete endothelial cell medium).

3.1.4 Remove a cryo vial of cells from the liquid nitrogen tank. Keep on ice.

NOTE: The cells were grown, and a seed lot was made as described by the manufacturer. The cell line is covered by a biological material transfer agreement. The cells are obtained at passage number 25 and should not be used further than passage 35.

3.1.5 Thaw rapidly in a 37 °C water bath.

3.1.6 Transfer the cells in the T75 Cell$^+$ flask and return the flask at 37 °C, 5% CO_2 and 95% humidity for 2 to 4 h.

3.1.7 Remove the medium carefully without losing or removing cells. Rinse the cells once with 10 mL of complete endothelial cell medium.

3.1.8 Add 15 mL of complete endothelial cell medium.

3.1.9 Culture cells at 37 °C, 5% CO_2 and 95% humidity until 100% confluence (not less than 4 days) without changing the medium.

3.2 Subculturing the hCMEC/D3 to prepare the insert of the BBB

3.2.1 Coat a new T75 Cell$^+$ flask or inserts with the type I Rat Collagen as described above (step 3.1).

3.2.2 Remove the medium from the flask. Rinse the cell monolayer with 10 mL of PBS supplemented with Ca^{2+} and Mg^{2+}.

3.2.3 Add 2 mL of trypsin-EDTA solution and incubate 5 min at 37 °C.

3.2.4 Shake the flask and make sure that the cells are fully detached from the plastic surface.

3.2.5 Add 4 mL of complete endothelial cell medium to inactivate the trypsin.

3.2.6 With a 5 mL plastic pipette, mechanically dissociate the cells by aspirating and flushing the cell suspension while maintaining the 5 mL pipette to the bottom of the flask at least 5 times.

3.2.7 Count the cells and use 5 x 10^4 cells/insert for a 12 well polyester membrane culture inserts insert and 2 x 10^6 cells for a T75 Cell$^+$ flask. Prepare also three filters without cells for the PE_{Ly} (i.e., endothelial cells permeability to Lucifer yellow see below paragraph 4.2) experiments with the Polyester membrane culture inserts.

3.2.8 Incubate the flasks or inserts at 37 °C, 5% CO_2 and 95% humidity until 100% confluence.

3.2.9 Do not change the medium for T75 Cell$^+$ flask until a new passage. On the contrary for BBB on polyester membrane culture inserts: change medium at days 2 and 4, use the BBB for experiments at day 6.

4. Construction and quality control of the BBB-Minibrain (Figure 2)

4.1 Setting up a BBB-Minibrain Polyester membrane culture insert device (**Figure 2B**)

4.1.1 Grow the hCMEC/D3 cells on 12 well Polyester membrane culture insert filters for 6 days

on endothelial cell medium before using them for the experiment.

4.1.2 Coat a 12 well plate with poly-D-lysine (1 mL/well, 10 µg/mL, 4 h at RT) and then laminin (1 mL/well, 1 µg/mL, overnight at RT).

4.1.3 Remove laminin and add 1 mL of endothelial cell medium supplemented with 5% FBS (5% endothelial cell medium).

4.1.4 Incubate for 1 h at 37 °C, 5% CO2 and 95% humidity.

4.1.5 Gently trypsinize the NT2-N/A (as described in step 1.3.9) and CHME/Cl5 (as described in step 2.2) and dissociate the cell pellets with complete endothelial cell medium.

4.1.6 Count the cells, mix 3.6 x 10^5 NT2-N/A and 0.4 x 10^5 CHME/Cl5 cells/well and seed the 12 well plate.

4.1.7 At T=24 h (24 h after seeding): change the used medium with fresh complete endothelial cell medium (Minibrain cells and endothelial cells) and transfer the hCMEC/D3 Polyester membrane culture insert filter on the top of the Minibrain cells. Incubate the BBB-Minibrain at 37 °C, 5% CO_2 and 95% humidity.

NOTE: The BBB-Minibrain will then consist of a layer of human endothelial cells hCMEC/D3 on filter isolating the luminal compartment (or "blood" compartment) and of a mixed culture of human cells hNT2-N/A and hCHME/Cl5 (Minibrain) at the bottom of the well defining the abluminal compartment (or "brain" compartment) (**Figure 2B**).

4.2 Validation of the endothelial permeability of the BBB-Minibrain (Quality Control) (**Figure 2C**)

4.2.1 Prepare the transport buffer (TB), which is HBSS (Hanks' Balanced Salt Solution) buffer with Ca^{2+} and Mg^{2+} supplemented with 10 mM HEPES and 1 mM sodium pyruvate.

4.2.2 Prepare 12 well plates with 1.5 mL of transport buffer per well.

4.2.3 At T= 0, make fresh transport buffer supplemented with 50 µM Lucifer Yellow (LY-TB).

4.2.4 Reverse each filter upside down to remove carefully the medium without affecting the endothelial cell barrier.

4.2.5 Place the filter on the filled 12 well plate and add 0.5 mL of LY-TB.

4.2.6 Incubate the plates in an incubator at 37°C, 5% CO_2 and 95% humidity.

4.2.7 At T=10 min transfer the filters on new TB filled 12 well plates and keep the abluminal compartment of the first plate for OD reading.

4.2.8 At T= 25 min, repeat the step 4.2.7.

4.2.9 At T= 45 min, stop the transport by removing the filters from the plates. Keep abluminal and luminal compartments for OD measures.

4.2.10 Transfer in a dark 96 well plates the samples: 10 µL with 190 µL TB for the LY-TB and the luminal compartments, 200 µL of sample for the abluminal compartments.

4.2.11 Measure the fluorescence of the LY-TB present in the different samples at λ428 nm λ535 nm (excitation and emission length waves respectively).

4.2.12 Calculate the endothelial permeability (Pe) toward LY-TB (Pe_{LY}) according to Siflinger-Birnboim, A et al. (1987)[39] and Da Costa, A et al. (2018)[34] by using the formula:

$1/PSe = (1/PSt) - (1/PSf)$ and $Pe_{LY} = PSe/S$.

NOTE: PSf is the permeability of the filter without cells, PSt is the permeability of the filter with cells, PSe is the permeability of the endothelial monolayer time the surface of the monolayer, S is the surface of the monolayer (for a 12 well filter=1.12 cm^2), Pe_{LY} is expressed in cm/min. For hCMEC/D3 the Pe_{LY} should be between 0.7 and 1.2 x 10^{-3} cm/min depending mainly of the FBS used in the experiments. Important: a Pe_{LY} higher than 1.2 means that the BBB is not tight enough and some leakage can be observed. Discard these barriers.

5. Use of BBB-Minibrain to highlight the presence of neuro-invasive viral particles in a Yellow Fever Virus vaccine sample, the French Neurotropic virus, YFV-FNV [34] (Figure 3)

5.1 BBB crossing and multiplication of Yellow fever viruses in the Minibrain

5.1.1 Use the BBB-Minibrain prepared as described in step 4.1.7 24 h before the addition of the virus; replace the medium by 2% FBS endothelial cell medium.

NOTE: It is extremely important to avoid changing the medium just before adding the virus. Changing the medium can activate the human endothelial cells and transiently open the barrier which will allow the passage of the virus. Here the medium is changed 24 h before starting the experiment.

5.1.2 At T= 0, add 3500 Plaque Forming Units (PFU) of YFV-FNV diluted in 50 µL of 2% FBS endothelial cell medium very carefully on the top of the luminal compartment. The control BBB-Minibrain is inoculated with 50 µL of 2% FBS endothelial cell medium without virus. Determine Pe_{LY} on companion well.

5.1.3 Incubate the BBB-Minibrain in an incubator at 37 °C, 5% CO_2 and 95% humidity.

5.1.4 After24 h, remove the polyester membrane culture inserts filter device and determine Pe_{LY}, sample 1 mL from the abluminal compartment and titrate the virus as described by A. da Costa et al. (2018)[34]. Replace the medium with fresh 2% FBS endothelial cell medium.

5.1.5 Incubate the BBB-Minibrain at 37 °C, 5% CO_2 and 95% humidity.

5.1.6 After 72 h, sample the medium from the abluminal compartment and titrate the virus, and/or extract the RNA from the Minibrain cells for gene expression analysis as described by A. da Costa et al. (2018)[34].

5.2 Amplification of neurotropic variants of YFV-FNV on Minibrain cells by serial passages

5.2.1 Use Minibrain cells coated 12 well plates (i.e., steps 4.1.6 and 4.1.7).

5.2.2 At time point 0 h, add 3500 Plaque Forming Units of YFV-FNV diluted in 50 µL of 2% FBS endothelial cell medium very carefully on the top of the cells (luminal compartment).

5.2.3 After 1 h, remove the medium with the virus inoculum and replace with fresh 2% FBS endothelial cell medium.

5.2.4 After 48 h, sample 500 µL of the culture medium and infect fresh Minibrain cells

5.2.5 After 120 h, sample 500 µL of the culture medium and infect fresh Minibrain cells.

5.2.6 After 192 h, save culture medium (=virus stock enriched) and extract the RNA from the Minibrain cells for gene expression analysis as described by A. da Costa et al. (2018)[34].

6. Use of BBB-Minibrain to study BBB crossing and brain cell targeting of a biomolecule

6.1 Transport across the BBB of a neuron targeting biomolecule

6.1.1 Use the Minibrain-BBB prepared as described step 4.1.7.

6.1.2 At time point 0 h, add the biomolecule (In the example provided in the result section, 58.75 ng/BBB of the cell permeant NeuroTag-NV molecules were added per BBB-Minibrain insert.

6.1.3 Incubate the BBB-Minibrain at 37 °C, 5% CO_2 and 95% humidity.

6.1.4 After 24 h, remove the polyester membrane culture inserts filter device and determine Pe_{LY}, then stain the Minibrain cells for hNeurons neurofilament Nf200 and detect the presence of the biomolecule in the Minibrain (In the example provided in the result section, NeuroTag-NV was detected using an antibody directed against the Strep-Tag it contains[35,40]).

6.2 Transport across the BBB of a neuroregenerative biomolecule and subsequent

neuroprotective assay in the Minibrain after the biomolecule has crossed the BBB

6.2.1 Use the Minibrain-BBB prepared as described in step 4.1.7.

NOTE: For molecules targeting neurons only, Minibrain cells can be replaced by the Neurons cells (NT2-N) only[38].

6.2.2 At time point 0 h, add 58.75 ng/BBB-Minibrain well of the cell permeant NV molecules (active form CPM-NeuroTag-NV, non-active form CPM-NeuroTag-NVΔ) described by C. Prehaud et al. (2014)[35].

6.2.3 Incubate the BBB-Minibrain in an incubator at 37 °C, 5% CO_2 and 95% humidity.

6.2.4 After 4 h, make individual wounds with an injection needle (26GX1/2", 12-4.5, Terumo, Belgium) on the Minibrain cells. Make at least 10 scratches on each individual well. Determine Pe_{LY} on the companion well.

6.2.5 Incubate the BBB-Minibrain in an incubator at 37 °C, 5% CO_2 and 95% humidity.

6.2.6 After 8 h, replace the medium in the abluminal compartment by fresh complete endothelial cell medium.

NOTE: It is important to replace the medium at this step since the wounding of the Minibrain cells can lead to cell death and then the release of cytotoxic compounds.

6.2.7 Incubate the BBB-Minibrain in an incubator at 37 °C, 5% CO_2 and 95% humidity.

6.2.8 After 48 h, stained the cells for axon regeneration of hNeurons as described by C. Prehaud et al. (2013)[40].

REPRESENTATIVE RESULTS:

The BBB-Minibrain is an in cellulo experimental model of blood-brain interface.

The BBB-Minibrain is set up on the polyester membrane culture insert system to mimic a blood compartment on the upper level and a brain compartment on the lower level of the blood-brain interface (**Figure 2A,B**). It consists of a luminal compartment with the hCMEC/D3 endothelial cells on the filter forming the BBB and an abluminal compartment, which contains the Minibrain human tri-culture of cerebral cells (neurons, astrocytes and microglial cells). The Minibrain cells exhibit a classical tri-culture mixed population phenotype (**Figure 1,** lower right panel) and express specific markers of each type of cell as shown by da Costa A. et al., 2018[34].

The use of hCMEC/D3 layer in the BBB-Minibrain allows obtaining a strong barrier with a mean Pe_{LY} of 0.95e-03 cm/min and a mean Trans Endothelial Electrical Resistance (TEER) of 51.89 Ω/cm^2. These values are in the range of the best values ever described for a human endothelial cell line[27,41] in such a polyester membrane culture insert system (**Figure 2C**). These cells express

tight junction protein markers such as ZO-1 and cadherin as expected by the permeability quantification (data not shown). They also express all the subsets of receptors, efflux transporters or transporters [Receptors: LDLR, low density lipoprotein receptor; LRP1, low density lipoprotein receptor related protein 1; INSR, insulin receptor; LEPR, leptin receptor; LU, basal cell adhesion molecule; TFRC, CD71 antigen; AGER, advanced glycosylation end-product receptor. Efflux transporters: ABCB1 (P-gp); ABCG2 (BCRP); ABCC1 (MRP1); ABCC2 (MRP2); ABCC4, ABCC4 protein; ABCC5, ABCC5 protein. Transporters: STRA6, stimulated by retinoic acid gene 6 protein; SLC2A1, glucose transporter type 1; SLC7A5, large neutral amino acid transporter 1; SLC1A1, solute carrier family 1 protein; SLC38A5, solute carrier family 38 member 5 protein; SLC16A1, monocarboxylate transport protein 1], which are key relevant proteins for their biological functions (**Figure 2D**)[21].

The BBB-Minibrain allows selecting, amplifying and characterizing rare neuroinvasive viral variants from a live virus vaccine preparation.

The BBB-Minibrain culture device was used as an in cellulo test allowing isolation and amplification of rare neuroinvasive/neurovirulent variants potentially present in Yellow Fever viruses (YFV) viral live vaccines[42]. YFV is a viscerotropic virus targeting the liver that does not efficiently cross the BBB. The French neurotropic virus, FNV, was used as a live YFV vaccine until the 1980s[33,43]. FNV was found to cause post-vaccinal neuropathogenesis in children (0.3 to 0.4% among vaccinees) and thus was discontinued in 1982[33,43]. We used FNV here as a prototype of YF virus which may contain a high proportion of neuroinvasive/neurovirulent variants[42,44]. A FNV virus preparation (i.e., 3500 PFU/ml) was added in the luminal compartment of a BBB-Minibrain, the control was a BBB-Minibrain that was mock-infected. Presence of the virus particles in the luminal compartment does not alter the barrier permeability as measured 24h later since the Pe_{LY} in the viral wells was not different from the Pe_{LY} of control wells ($0.80 \pm 0.09 \times 10^{-3}$ cm/min and $0.86 \pm 0.09 \times 10^{-3}$ cm/min for Pe_{LY} in the control and YFV-FNV wells respectively). The content of the abluminal compartment was evaluated for the presence of virus by plaque assay, at 24 h post infection. The Minibrain cells were incubated two days further to evaluate the amplification of neuroinvasive variants in brain cells and to monitor gene expression as described on the cartoon shown on **Figure 3A**.

We detected some YFV-FNV viruses, which can cross the BBB at 24 h (mean 43 PFU/mL). The viruses were amplified for the next two days by the multiplication of the virus inside the brain cells (mean titer of 4.69×10^4 PFU/mL), (**Figure 3B**). Of note, a vaccine strain preparation which does not cause post-vaccinal neuropathogenesis would not efficiently cross the BBB in this system as it has been demonstrated by da Costa A. et al. (2018)[34]. We identified two biomarkers of the multiplication of the virus: The Interferon Stimulated Gene 15 (ISG15) and the Interferon Regulatory Factor 7 (IRF7). The expressions of these two biomarkers were stimulated when this neuroinvasive viral population was serially passaged on Minibrain cells (**Figure 3C**). These upregulations measured by Q-RT-PCR were strictly correlated to the viral load (Pearson p value 0.0016 for *ISG15* and 0.0260 for *IRF7*).

BBB-Minibrain allows both assaying the ability of a biomolecule to pass the barrier and at the same time testing whether the biomolecule function was preserved after BBB crossing.

NV is a biomolecule derived from Rabies virus which possesses astonishing properties of neuroprotection and neuroregeneration[40]. This small polypeptide that neither naturally crosses the cell membrane nor the BBB was fused with a CPM able to target the neurons. Our choice was to use the variable part (VHH) of a single chain antibody of Llama that crosses the biological membranes including the BBB[36,45] and a NeuroTag targeting neurons specifically. NV was linked to the VHH and a NeuroTag, to construct a CPM-NeuroTag-NV (**Figure 4A**) and to the VHH only to construct a CPM-NeuroTagΔ-NV lacking the specific NeuroTag allowing the targeting of the Neurons (**Figure 4B**). After being added to the luminal compartment, CPM-NeuroTag-NV (green dots) crosses the BBB and was able to target human neurons (in red) (**Figure 4C, D**). CPM-NeuroTagΔ-NV crosses the endothelial cell barrier (green dots) but targets less efficiently the human neurons (majority of green dots are located outside the neurons) (**Figure 4D**).

As described above, NV was linked to a VHH and a NeuroTag to construct a CPM-NeuroTag-NV. We did the same for NVΔ an inactive form of NV lacking the active part of NV (CPM-NeuroTag-NVΔ) (**Figure 5A**). After being added to the Luminal compartment, CPM-NeuroTag-NV crosses the BBB and was able to regenerate axons of human neurons after wounding (**Figure 5B**, right panel), on the contrary to CPM-NeuroTag-NV Δ the inactive form of NV (**Figure 5B**, left panel)[40]. These properties were assayed in two types of protocols; either in a pre–exposure (**Figure 5C**) or in a post exposure protocol (**Figure 5D**). When applied before the axon scratching (4 h, H4), CPM-NeuroTag-NV triggers the neuroprotection of the wounded neurons and the axonal regeneration on the contrary of the mock treated cells (i.e., control) or the cells treated with CPM-NeuroTag-NVΔ (mean regeneration 91% and 12%-10% respectively, **Figure 5C**). When the biomolecule was applied after the axonal lesions (1 hour, H1, **Figure 5D**) in order to mimic a therapeutically post-exposure protocol, the CPM-NeuroTag-NV is still able to regenerate axons on the contrary of the disabled form of CPM-NeuroTag-NV Δ, (mean regeneration 85% and 5.1% respectively) (**Figure 5D**).

FIGURE AND TABLE LEGENDS:

Figure 1: The Minibrain model. Time table of the NT2-N/A and CHME/Cl5 cultures (upper panel). Photographs (lower panels) of the original cell line (Ntera/cl2.D1) in the left panel, the pseudo neurospheres obtained after ATRA treatment in the middle upper panel , the CHME/Cl5, in the middle lower panel and the Minibrain triculture (Cristal Violet staining), in the right panel, resulting of the mixture of NT2-N/A and CHME/CL5 cultures. Scale bar 100 µm.

Figure 2: the BBB-Minibrain model. A. Time schedule of the hCMEC/D3 cultures and photograph of the BBB-Minibrain device: a polyester membrane culture inserts-filter with hCMEC/D3 endothelial barrier is held with a forcep before to be inserted in to one well of a 12 wells cell culture plate. **B**. Cartoon describing the device with the luminal (Blood) compartment containing the endothelial cell barrier and the abluminal (Brain) compartment containing the Minibrain cells (human neurons, astrocytes and microglial cells). **C**. Representative measures of the BBB-Minibrain permeabililty by TEER (i.e., TransEndothelial Electrical Resistance) on three devices or permeability to LY (i.e., Pe_{LY}) on five filters. **D**. expression analysis by q-RT-PCR of the Receptors, efflux Transporters and Transporters on the endothelial cells hCMEC/D3.

Figure 3: **The BBB-Minibrain model allows identification of neuroinvasive variants among live YFV vaccine preparation. A**. Schedule of the experiment. **B**. Quantification of the viruses which have crossed the BBB by plaque forming unit (PFU) titration of live virus (each point represents one polyester membrane culture inserts experiment). **C**. Plot of *ISG15* and *IRF7* gene expression measured by q-RT-PCR as a function of the number of viral particles in the viral load.

Figure 4: The BBB-Minibrain allows testing of neuroregenerative biomolecule properties: specific targeting of neurons when NV is fused to NeuroTag. A. Cartoon of the CPM based NV (i.e., CPM-NeuroTag-NV) containing a specific NeuroTag to target neuron specifically. **B**. Cartoon of the CPM based NV deleted of the NeuroTag (i.e., CPM-NeuroTagΔ-NV). **C**. Representative immunofluorescence photographs of the human neurons. Scale bar 50 µm. NF 200 in red, CPM-NeuroTag-NV/CPM-NeuroTagΔ-NV in green, nuclei in blue. **D**. CPM-NeuroTag-NV molecules can cross the BBB and target human neurons more efficiently than CPM-NeuroTagΔ-NV. Infected neurons and total population of neurons were counted on triplicate slides after immunolabeling. Total neurons correspond to NF200 positive cells (in red). Any red cells associated with one or more green dot (CPM-NV) is counted as a positive neuron. Unpaired student's t-test two tailed ****p=0.0002*.

Figure 5: The BBB-Minibrain allows testing of neuroregenerative biomolecule properties: BBB crossing does not alter the neuroregenerative properties of NV. A. Cartoon of the CPM based NV (i.e., CPM-NV) and its disabled counterpart (i.e., CPM-NVΔ). **B**. Axon regeneration by CPM-NV in an *in cellulo* scratch assay (right panel). CPM-NVΔ cannot trigger axon regeneration (left panel), scale bar 100 µm. **C**. CPM-NV can cross the endothelial cell and regenerate axons on the neurons of the BBB-Minibrain when applied 4 h before the lesion: (upper panel) scheme of the experiment; (lower panel) quantification of the regeneration. **D**. CPM-NV is also active in a therapeutically protocol when it is applied 1 h after the wounding: (upper panel) scheme of the experiment; (lower panel) quantification of the regeneration. Unpaired student's t-test two tailed ****p<0.0001*. Regeneration was calculated from triplicate experiments (>800 neurons counted) after staining of the neuronal cells.

DISCUSSION:

In this article we demonstrated how to build an in cellulo blood/brain interface, the BBB-Minibrain, by combining a BBB model and a culture of mixed brain cerebral cells (Minibrain) into a single kit. This system is biologically relevant, easy to set up and handle for experimenters well trained in cell culture.

As for any other in vitro model of BBB, reliable results would be obtained if drastic control of tightness of the barrier is applied. Inserts should be carefully tested for permeability and any insert with inadequate permeability values (i.e., a Pe_{LY} higher than 1.2) should be discarded.

FBS samples should be carefully tested to identify a batch that does not disable the characteristics of the BBB. The same advice can be made regarding the vehicle medium in which virus particles or biomolecules are diluted. It is also recommended to keep as small as possible the volume of

biomolecule or virus suspension, in order to not alter the medium composition of the luminal compartment.Differentiation of human co-culture neuron/astrocytes culture from the Ntera/CL2.D1 is a proven technology. In contrast to human neurons which are post-mitotic, astrocytes are still dividing cells. High proliferation of the astrocytes cells is sometimes observed. If this happens, the Minibrain culture has to be discarded. The CHME/Cl5 cells we used in our laboratory were phenotyped to prove that they were of human origin (*Homo sapiens* CCNT1 phenotyping). There is a risk that some CHME lots used in some laboratories may be of rat (*Rattus norvegicus*) origin as claimed by Garcia-Mesa Y. et al. (2017)[46]. Therefore, it is recommended to check for the origin of the CHME/Cl5 cell line.

The Minibrain human tri-culture system including post mitotic neurons (mainly dopaminergic), astrocytes and a microglial cell line, mimics a simplified cerebral environment. This system can still be improved. One can imagine adding oligodendrocytes to the culture with the goal to obtain myelinated axons or primary microglial cells. The minibrain can also be replaced with a mixed culture of human-derived stem cells[19-21]. Nevertheless, the variability between different lots of cells will have to be carefully mastered. The BBB-Minibrain complexity can also be increased by adding pericytes[23]. In our hands, this improvement was impeded by the difficulty to have reliable access to pericytes of human origin.

We have demonstrated the feasibility and usefulness of this kit mimicking the blood-brain interface, to i) isolate rare neuroinvasive virus particles from a Yellow Fever vaccine sample that have evolved the property to enter the brain through the BBB, and ii) amplify these neuroinvasive sub-populations in the Minibrain tri-culture mimicking a simplified brain parenchyma. Future applications can be to extend the quality control of other live vaccines such as the mumps vaccine and to promote the BBB-Minibrain as a mean to study neurovirulence features in vitro.

The second pilot study was to show that a drug candidate passes through the BBB and reaches the Minibrain, without losing its neuro-regenerative properties. We are strongly convinced that the BBB-Minibrain can allow major advances in the pre-sorting of molecules before releasing them to preclinical tests. The use of the BBB-Minibrain should facilitate the implementation of 3Rs measures aimed at reducing the use of animal testing for both reglementary assays and experimental research.

Altogether with the next development of *in silico* approaches (computer model), we will soon be able to identify drug candidates with a high probability of crossing the BBB, the development of a 3D model In cellulo mimicking the nervous parenchyma blood interface would be of great help.

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DISCLOSURES:

The intellectual property of the system was the patents referenced in 32, 35 and 38.

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Figure 1

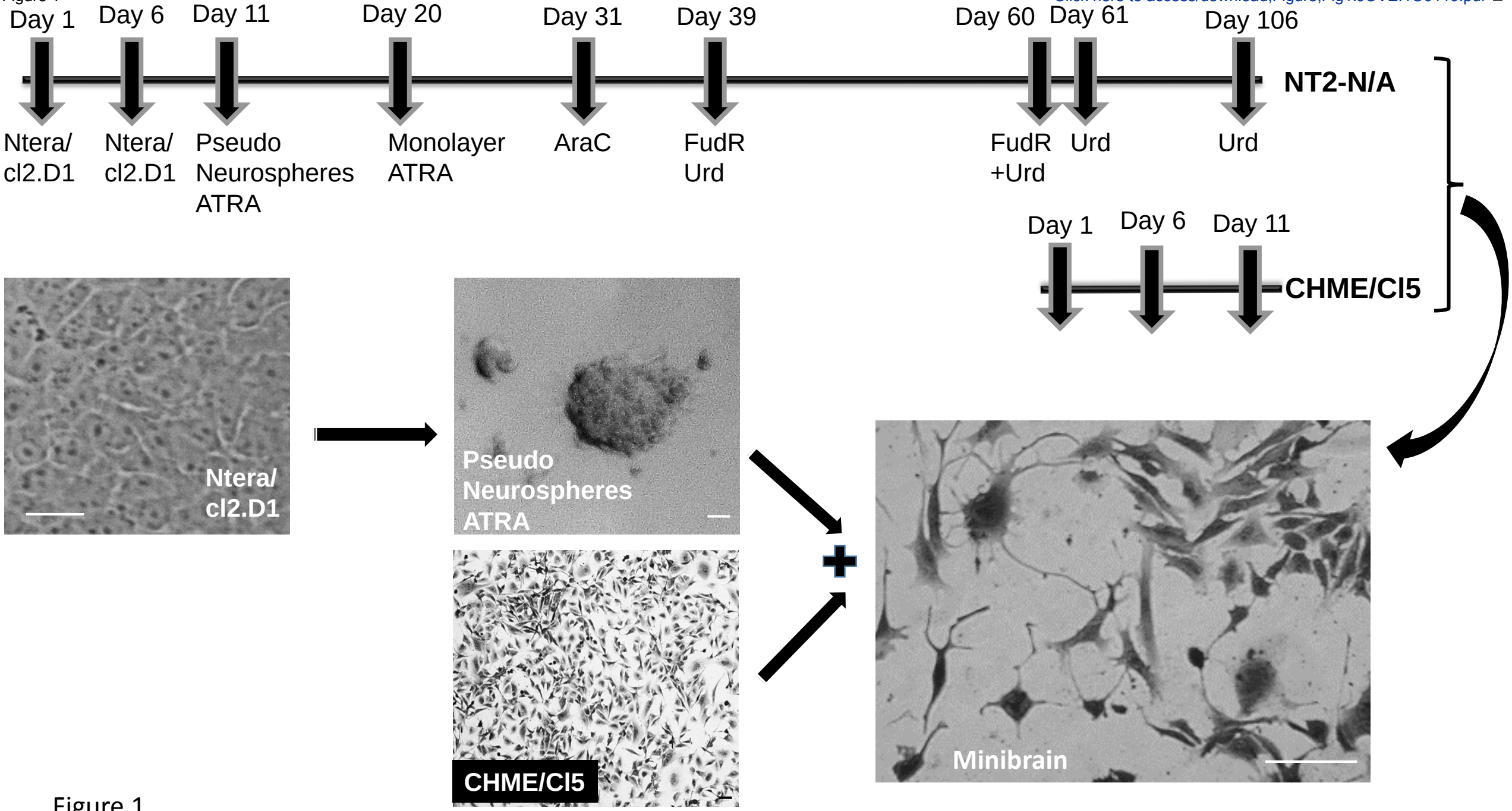


Figure 1

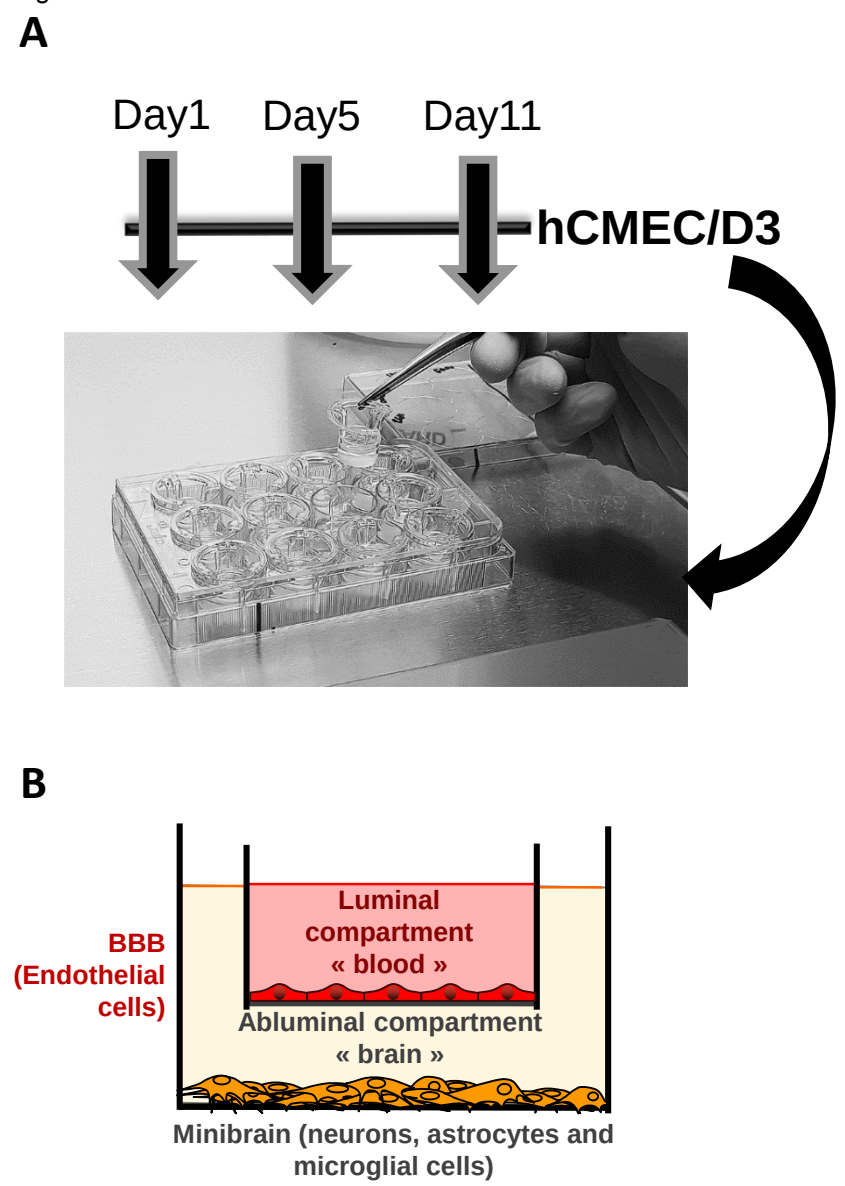
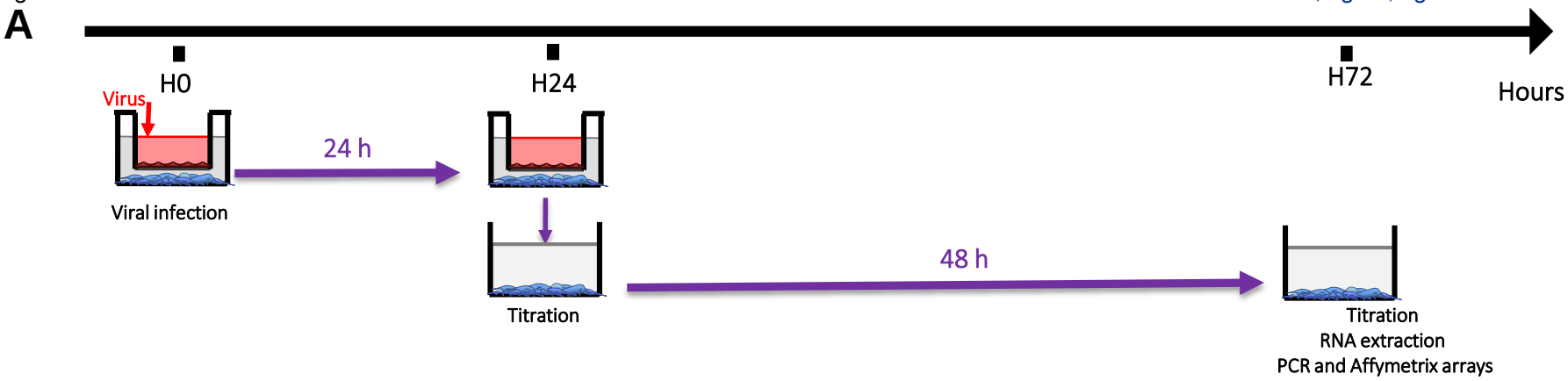
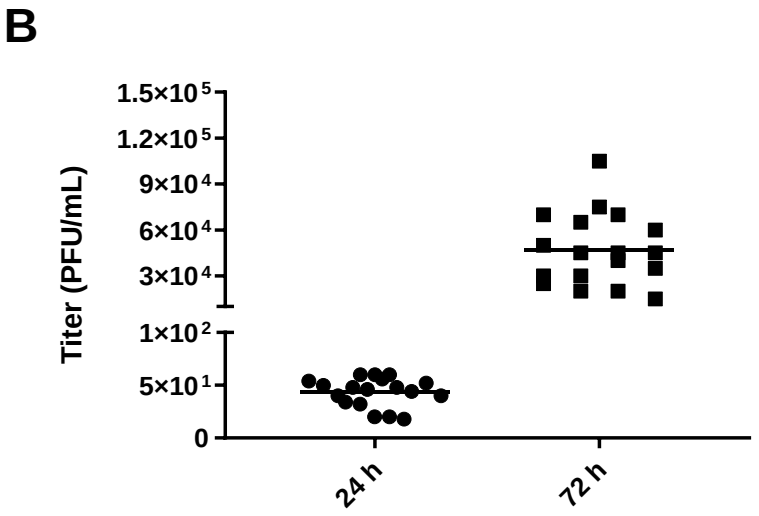


Figure 2

Figure3





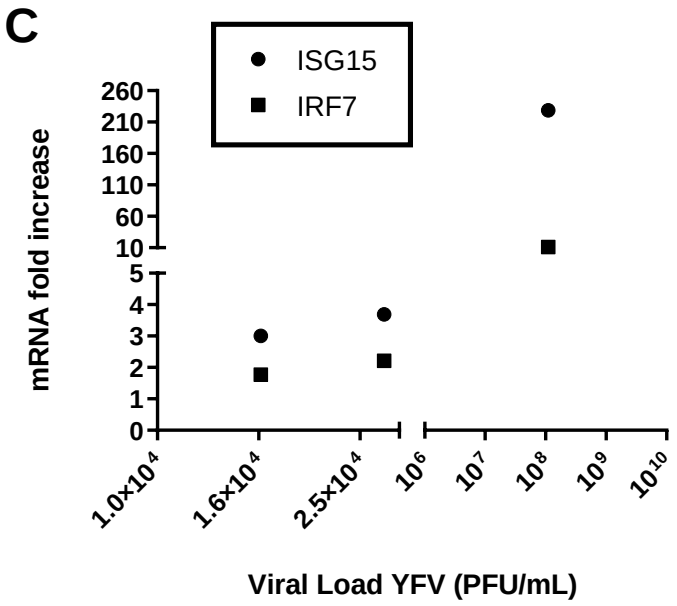


Figure 3

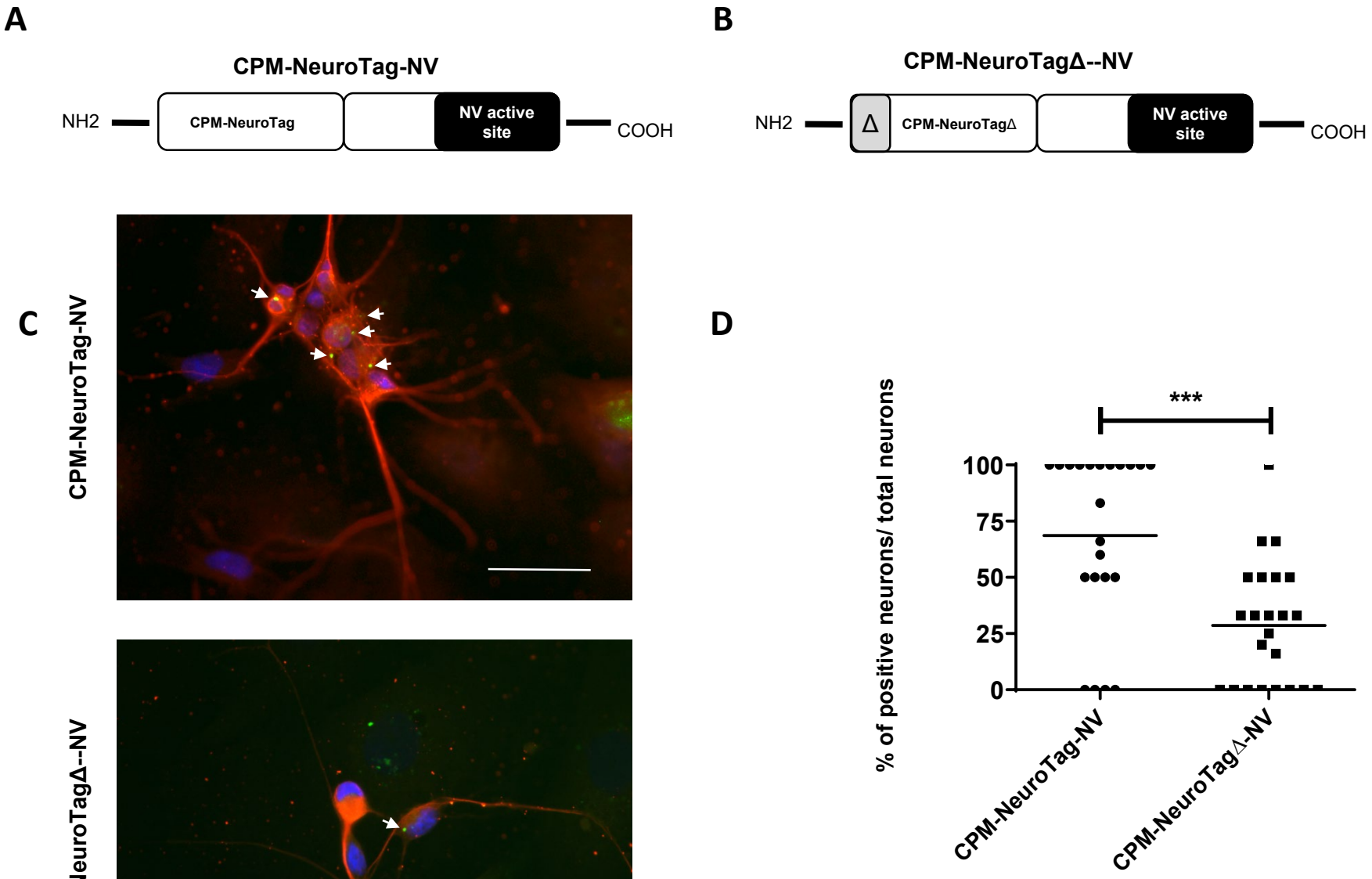


Figure 4

Figure 5

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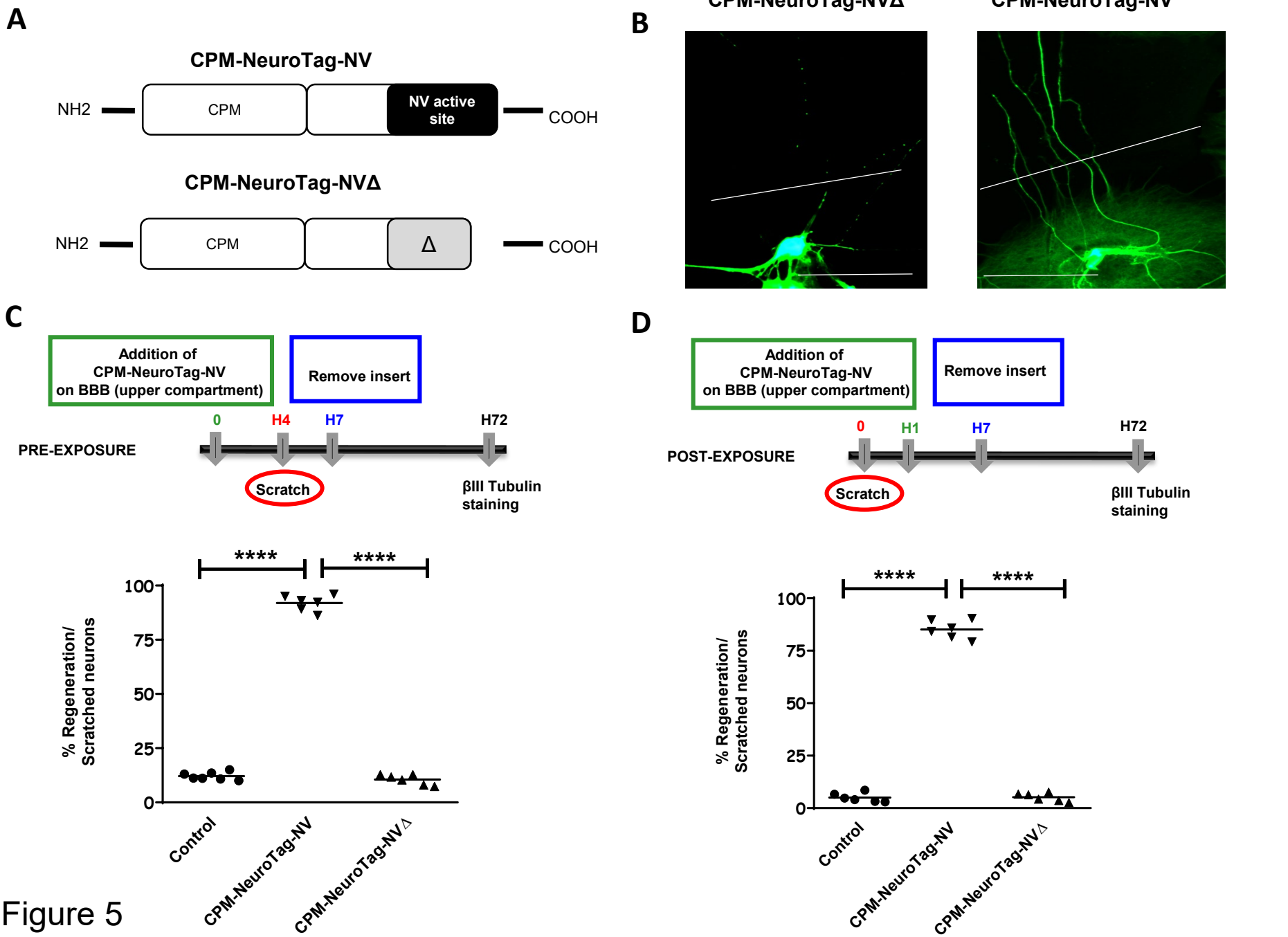

Figure 5

Name of Material/ Equipment	Company	Catalog Number
12 well plates	Corning	3336
5-fluoro-2'deoxyuridine	Merck-Sigma Aldrich	F0503
85mm Petri Dish	Sarstedt	83-3902-500
Anti-Nf200	Merck-Sigma Aldrich	N4142
β-mercapto-ethanol	Merck-Sigma Aldrich	M3148
CHME/Cl5	Unité de Neuroimmunologie Virale	On request to Dr Lafon
CMC	Calbiochem	217274
Cytosine β-D-arabinofuranoside	Merck-Sigma Aldrich	C1768
Dark 96 well plates	Corning	3915
DMEM F12	Thermofisher Scientific	31330-038
DMSO	Merck-Sigma Aldrich	D2650
Endogro IV	Millipore	SCME004
Ethanol	Carlo Erba	529121
FBS	Hyclone	SV30015-04
Formaldehyde	Merck-Sigma Aldrich	252549
GIEMSA	RAL Diagnostic	320310
Goat-Anti Mouse	Jackson Immuno Research	115-545-003
Goat-Anti Rabbit	Thermofisher Scientific	R37117
HBSS with Ca2+-Mg2+	Thermofisher Scientific	14025-100
hCMEC/D3	Cedarlane	CLU512
Hepes 1M	Thermofisher Scientific	15630-070
Hoescht 33342	Merck-Sigma Aldrich	33263
Laminine	Merck-Sigma Aldrich	L6274
L-glutamin	Thermofisher Scientific	25030-024
Lucifer Yellow	Merck-Sigma Aldrich	L0259
MEM 10X	Thermofisher Scientific	21430
MEM 1X	Thermofisher Scientific	42360
Ntera/Cl2D.1	ATCC	CRL-1973
Paraformaldehyde	Electron Microscopy Sciences	15714
PBS without Ca2+-Mg2+	Thermofisher Scientific	14190
PBS-Ca2+-Mg2+	Thermofisher Scientific	14040-091

Pen/Strep	Eurobio	CXXPES00-07
Poly-d-Lysine	Merck-Sigma Aldrich	P1149
Prolong Gold	Thermofisher Scientific	P36930
Qiashredder	QIAGEN	79656
Rat Collagen I	Cultrex	3443-100-01
Retinoic Acid All-Trans	Merck-Sigma Aldrich	R2625
RNA purification kit	QIAGEN	74104
SDS	Merck-Sigma Aldrich	L4509
Sodium bicarbonate 5.6%	Eurobio	CXXBIC00-07
Sodium Pyruvate	Thermofisher Scientific	11360
T75 Cell+ Flask	Sarstedt	83-1813-302
Transwell	Corning	3460
Trypsin-EDTA	Merck-Sigma Aldrich	T3924
Ultra Pure Water	Thermofisher Scientific	10977-035
Uridine	Merck-Sigma Aldrich	U3750
Versene	Thermofisher Scientific	15040-033
YFV-FNV	IP Dakar	Vaccine vial

Comments/Description

endothelial cell medium

Tissue culture polystyrene flask with specific surface treatment (Cell+) for sensitive adherent cells

polyester porous membrane culture inserts

EDTA

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Title of Article: A human blood-brain interface model to study barrier crossing by pathogens or medicines

Author(s): da COSTA A, PREHAUD C ... et al.

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1. The editor has formatted the manuscript to match the journal's style. Please retain the same.

2. Please address all the specific comments marked in the manuscript.
3. For the protocol section, please make the steps crisp, and remove the redundancy to bring out the clarity.
4. Please use imperative tense throughout as if describing someone how to perform your protocol. Please use complete sentences.
4. Please proofread the manuscript well for any grammar or spelling issues.
5. Once the protocol is formatted please ensure that the highlight is no more than 2.75 pages including headings and spacing and is in alignment with the title of the manuscript. Please ensure that the highlight forms a cohesive story.
5. Please ensure that all the microscopic figure panels have a scale bar.
6. Please write the discussion in a paragraph style including citations where ever applicable.

Answer to each editor' commentary are in the text.

The comment on references style asking to provide the full name of journals cannot be corrected with the Jove endnote style you provided. This Jove endnote style still give journal abbreviations. There is likely a bug in this application.

Best regards

Monique Lafon