University of Szeged
Faculty of Science and Informatics
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THE ROLE OF BRAIN ENDOTHELIAL SURFACE CHARGE AND GLYCOCALYX IN THE FUNCTION AND INTEGRITY OF THE BLOOD-BRAIN BARRIER

Summary of the Ph.D. thesis

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1. Introduction and aims

The blood brain barrier (BBB) is a dynamic interface that separates the blood from the central nervous system (CNS). The microvessels that form the BBB are composed of brain endothelial cells (ECs) that exhibit special features and form a functional unit with the neighboring pericytes, astrocytes and neurons. The BBB is responsible for keeping the homeostasis of the CNS, providing nutrients for proper cellular functions and for the protection of the brain. These are regulated by the specific features of the brain ECs: by tight intercellular junctions, lack of fenestrae, low level of transcytosis and polarized expression of influx and efflux transporters. Besides the efflux transport systems and tight junctions, the endothelial surface glycocalyx (ESG), composed of glycoproteins and proteoglycans, represent another important defense element of the BBB (Figure 1).

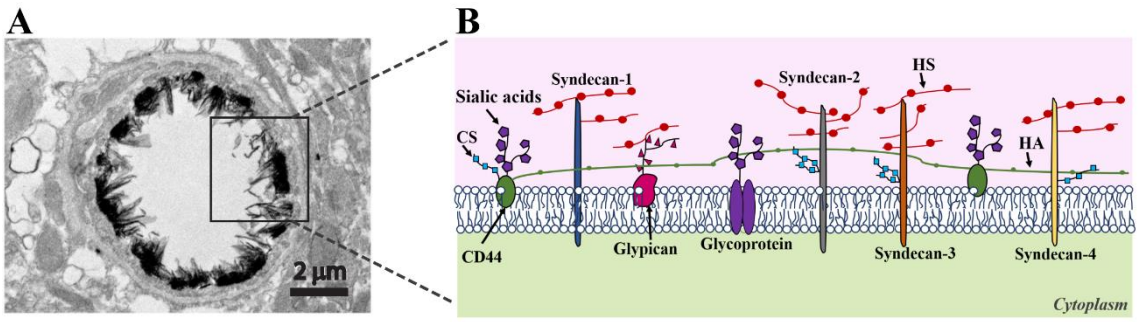

Figure 1. Illustration of the brain endothelial surface glycocalyx (ESG). (A) Transmission electron micrograph of cerebral continuous capillaries with lanthanum nitrate staining (Ando *et al*., 2018). (B) Drawing representing ESG core proteins, glycoproteins and glycosaminoglycans. HA: hyaluronic acid, HS: heparan sulfate, CS: chondroitin sulfate.

This negatively charged sugar-protein matrix on the surface of the brain ECs together with the negatively charged plasma membrane phospholipids act as a physical barrier for charged molecules, drugs and transmigrating cells. Despite of the potential importance of the negative surface charge in both physiology and pathology of the BBB, this area is under researched.

To reach our main goal, to study how changes in surface charge may affect the functions of brain endothelial cells and BBB integrity we used cell culture models of the BBB (Figure 2). *In vitro* BBB models are important tools to study barrier functions, pathologies, microbe, cell and drug interactions. In this study we aimed to compare three of these BBB models: (i) our primary rat brain EC, pericyte and astrocyte co-culture

model, (ii) the human hCMEC/D3 brain EC line, and (iii) a stem cell-derived human EC and bovine pericyte co-culture model (Figure 2). We wanted to compare the barrier integrity of these BBB models on cell culture inserts or in our patented lab-on-a-chip (LOC) device. We studied the integrity, the glycocalyx and surface charge of these BBB models in both setups.

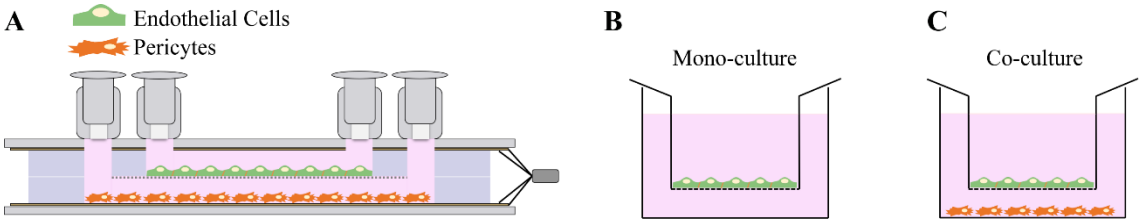

Figure 8. Illustrations of the human BBB model on lab-on-a-chip (LOC) device and cell culture insert. (A) Cross section of the LOC with the human BBB co-culture model: human endothelial cells (ECs) on the top of the membrane and brain pericytes in the bottom channel. (B) Mono-culture of human ECs in the cell culture insert. (C) The co-culture model in cell culture insert with human ECs on the top of the membrane and brain pericytes on the bottom of the culture well.

To investigate how the modulation of the glycocalyx with the neuraminidase enzyme or the membrane-intercalating lidocaine drug changes the surface charge and barrier integrity of brain ECs the rat co-culture model and the hCMEC/D3 cell line were used. Our further goal was to characterize the human brain like EC (BLEC) co-culture model under dynamic conditions for the first time by performing functional experiments and gene expression analysis.

2. Methods and techniques

1. Primary cell cultures: Organ harvest from rats was performed according to the regulations of the 1998. XXVIII. Hungarian law and the EU Directive 2010/63/EU about animal protection and welfare. For primary cell isolations brain tissues were obtained from 4-week-old and 1-day-old Wistar rats (Harlan Laboratories, United Kingdom) of both sexes. Experiments with lidocaine and neuraminidase were performed on primary rat brain endothelial cell triple co-culture models of the BBB consisting of primary rat brain endothelial cells and glial cells. These three cell types were isolated and cultured according to the method described in our previous studies (Nakagawa *et al.*, 2009; Veszelka *et al.*, 2013; Walter *et al.*, 2016, Barna *et al.*, 2020).

2. Other cell types: The human hCMEC/D3 brain endothelial cell line (Weksler *et al*., 2005), used as a simplified BBB model, was purchased from Merck Millipore. The cultures of hCMEC/D3 (passage number ≤ 35) were grown in special cell culture medium (Walter *et al*., 2016). This cell type was used in the lidocaine and neuraminidase experiments along with the primary cultures to study the possible modification of the surface glycocalyx. The other human BBB model was described by Cecchelli *et al*., in 2014. The BLEC model consists of stem cell derived human ECs (hECs) in co-culture with bovine brain pericytes, and was used in our biochip setup.

3. Biochip modeling: The lab-on-a-chip (LOC) device was built as described previously in Walter *et al*., 2016. To determine the importance of fluid flow on the human BBB model, the co-culture of human ECs with pericytes lasted for 6 days under static condition (Figure 3A) then 24 hours under dynamic condition (Figure 3B).

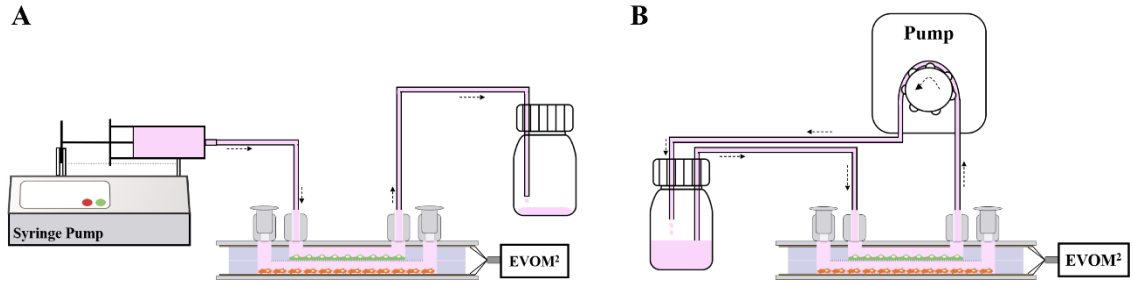

Figure 3. Illustration of the static and dynamic condition in the lab-on-a-chip device. (A) Static condition: a syringe containing the cell culture medium was placed in a syringe pump, which allowed automatic medium change through the top compartment every 8 hours (500 µl/min medium flow for 4 min). A reservoir was connected to the LOC to collect the discarded medium. (B) Dynamic condition: the device was connected to a peristaltic pump and a reservoir containing cell culture medium. Fluid flow was applied for 24 hours (flow rate 1 ml/min, shear 0.4 dyne/cm^2).

4. Viability assays: MTT and LDH assayss were done to test the effects of different concentrations of lidocaine and neuraminidase on hCMEC/D3 and primary ECs.

5. Barrier integrity measurements: Transendothelial electrical resistance (TEER) was measured both on cell culture inserts and the LOC device. The permeability of fluorescent markers with different charge and size across the barriers was evaluated. To complete these studies a P-glycoprotein efflux pump activity assay was also performed.

6. Morphological analysis: Phase contrast and fluorescent imaging was used to determine morphological changes of ECs. Junctional proteins were visualized by immunostaining, the surface glycocalyx by fluorescently labeled wheat germ agglutinin lectin.

7. Charge measurements: The zeta potential was measured by dynamic light scattering using a Zetasizer Nano ZS instrument (Malvern, UK). Surface charge of cells in suspension was measured after lidocaine or neuraminidase treatments or fluid flow.

8. MACE analysis: Massive analysis of cDNA ends was performed on total RNA samples. To compare gene expression differences between human BLEC/pericyte co-cultures in static and dynamic conditions MACE libraries were created and analyzed by bioinformatical methods.

3. Results

1. Comparison of the different cell culture models

To investigate the barrier properties of the BBB models on cell culture inserts or in the LOC device (Figure 4) we measured TEER and fluorescent marker permeability. The rat primary co-culture model was the tightest for ions on the cell culture inserts, but the BLEC model had the highest TEER in the LOC device. The weakest barrier properties were measured in both setups on hCMEC/D3 cells, which showed the lowest TEER and the highest permeability values compared to other BBB models (Figure 4 A-B).

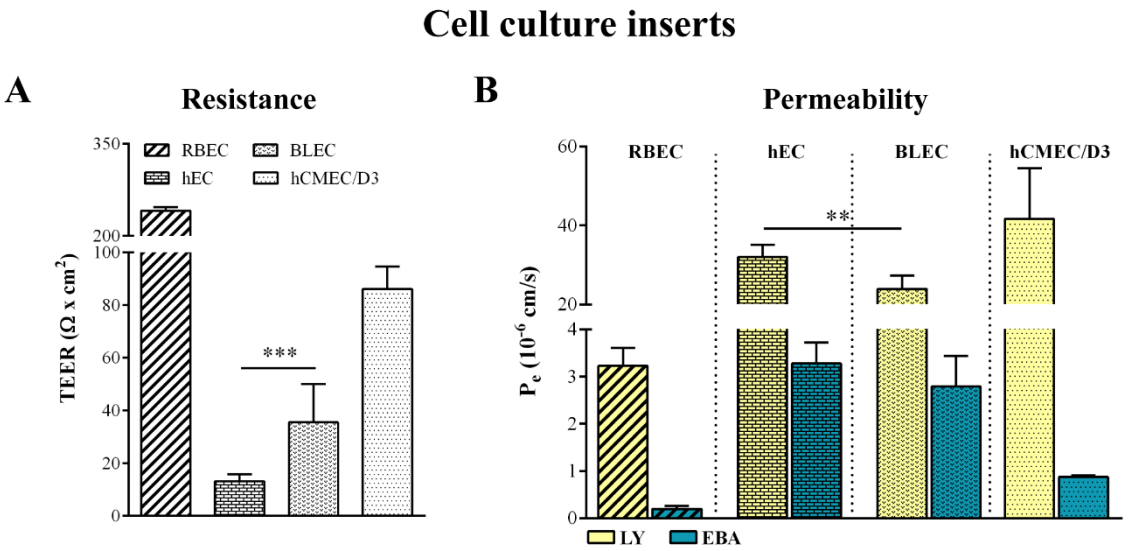

Figure 4 A-B Evaluation of the integrity of the blood-brain barrier (BBB) models kept on cell culture inserts. The following BBB models were used: hCMEC/D3 human brain endothelial cell line, a primary cell-based model consisting of rat brain endothelial cells (RBEC) co-cultured with rat pericytes and rat astroglia, human endothelial cells (hEC) in mono-culture, and brain like endothelial cells (BLEC) in co-culture with bovine brain pericytes. (A) Transendothelial electrical resistance (TEER) measurement on the BBB models cultured on cell culture inserts (n=3-12). (B) Endothelial permeability coefficients (P_e) of BBB models for Lucifer yellow (LY) and Evans blue labeled albumin (EBA) (n=3) Values are presented as mean ± SD on all graphs. **p<0.01, ***p<0.001 compared to hEC group. *(to be continued on the next page)***.**

We characterized the hEC mono- and BLEC/pericyte co-culture models in a LOC device for the first time. These stem cell derived models showed an increased TEER and a decreased permeability in the LOC compared to culture inserts (Figure 4 C-D).

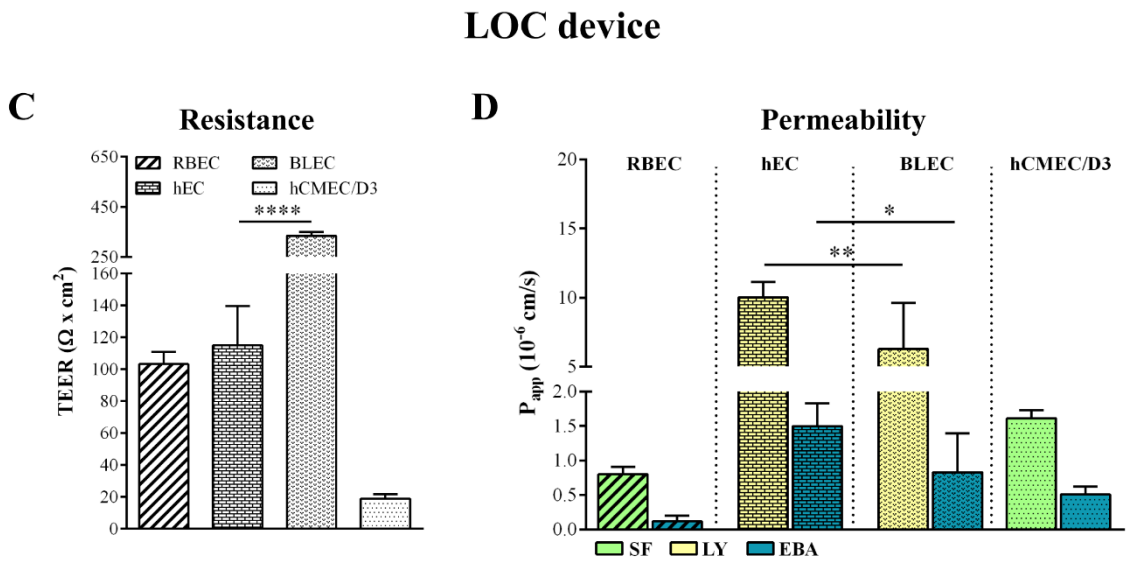

Figure 4 C-D. (C) TEER values of the BBB models cultured in the lab-on-a-chip (LOC) device (n=3). (D) Apparent permeability coefficient (P_{app}) of the BBB models for LY, EBA, and sodium fluorescein (SF) tracers (n=3-10). Values are presented as mean ± SD on all graphs.*p<0.05, **p<0.01, ****p<0.0001 compared to hEC group.

ESG could be visualized by lectin staining in all the BBB models cultured on inserts which showed an inhomogeneous staining pattern with higher intensity around the cell nuclei (Figure 5). We investigated for the first time the effect of co-culture on the glycocalyx of the stem cell derived human EC model using the LOC device, where we found a stronger staining, indicating a denser ESG in this setup.

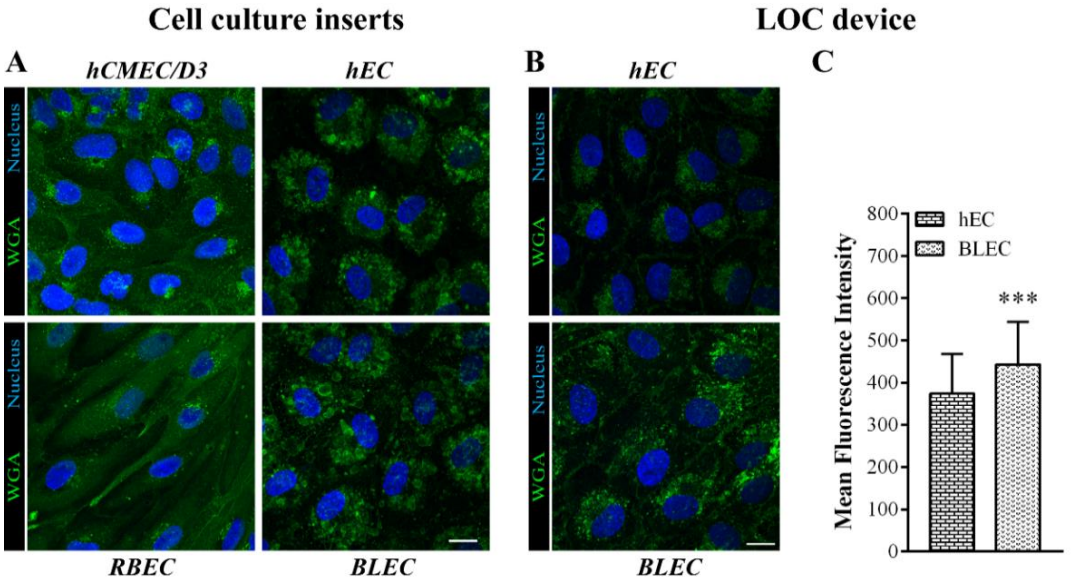

Figure 5. Staining of the cell surface glycocalyx of brain ECs with fluorescently labeled wheat germ agglutinin lectin (WGA). (A) BBB models on cell culture inserts. (B) Models on the LOC device. Green: glycocalyx. Blue: cell nuclei. Bar: 20 μm. (C) WGA staining analysis. ***, $p<0.001$ compared to the hEC mono-culture.

2. Modulation of brain endothelial surface charge by neuraminidase and lidocaine

Our next goal was to directly measure and modulate the surface charge of brain ECs. We tested two approaches. We applied neuraminidase enzyme, which specifically removes sialic acid residues from the ESG and therefore makes the surface charge more positive. We also tested lidocaine, a lipophilic and cationic therapeutic drug, which intercalates to plasma membranes, to change the surface charge of BBB models and study its effects on barrier properties and the permeability of charged molecules. In these experiments we used the hCMEC/D3 cell line as a simplified BBB model and the rat primary cell based co-culture BBB model. We directly measured the surface charge of hCMEC/D3 cells and primary rat brain ECs by laser Doppler velocimetry and found it highly negative. We demonstrated that neuraminidase decreased the amount of sialic acid residues on the glycocalyx and made the surface charge of brain ECs more positive (Figure 6). Cleavage of sialic acid residues from the ESG did not change the permeability of the BBB models for dextran and albumin.

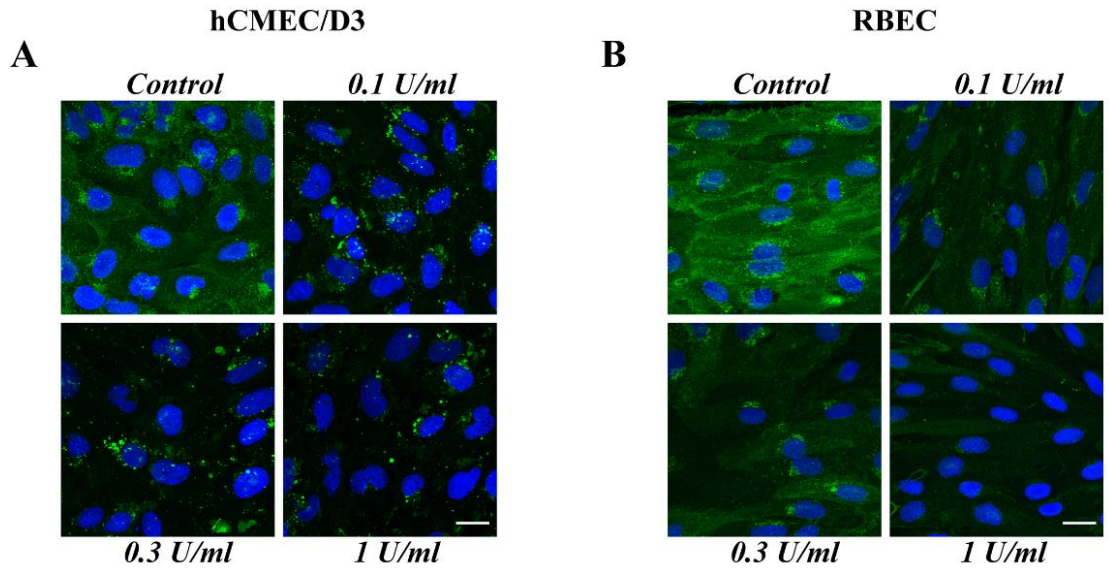

Figure 6. The effect of neuraminidase (0.1, 0.3 and 1 U/ml) on hCMEC/D3 human brain endothelial cells and rat primary brain endothelial cells (RBEC). (A and B) Representative pictures of the staining with wheat germ agglutinin (WGA) lectin labeled with Alexa 488 with or without neuraminidase treatment. Scale bar: 20 µm.

We revealed that lidocaine also changed the surface charge of brain ECs and made zeta potential values more positive in both BBB models. Lidocaine treatment slightly decreased the TEER of brain ECs suggesting an increased paracellular ionic permeability. However, there was no change in the permeability for negatively charged or neutral hydrophilic markers Lucifer yellow and dextran during lidocaine treatment. This result indicates that lidocaine may affect the paracellular pathway for small ions, but not for water-soluble larger molecules. In contrast, we found that the permeability of the cationic and lipophilic rhodamine 123 was decreased (Figure 7) suggesting an interaction of the cationic molecules lidocaine and rhodamine at the plasma membrane of brain ECs. We also proved that this effect is not based on an interference with efflux pump activity (Figure 7).

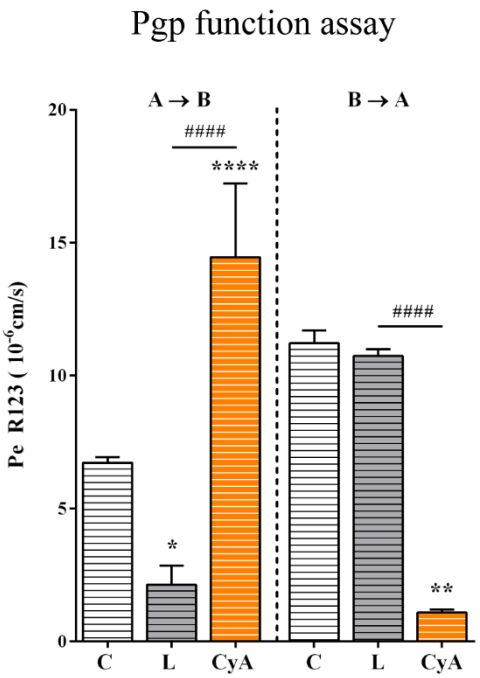

Figure 7. Effects of lidocaine on the P-glycoprotein efflux pump activity in primary rat brain endothelial cells (RBEC). Permeability of rhodamine 123 (R123) was measured from the luminal to the abluminal (A to B) and from the abluminal to the luminal (B to A) direction after 30 min treatment with lidocaine or with P-glycoprotein pump inhibitor cyclosporin A (CyA) (n=4). Values are presented as mean ± SD. Data were analyzed by one-way ANOVA followed by Bonferroni post-test. *p<0.05, **p<0.01, ****, ####p<0.0001, compared to the control and between groups. C: control, L: lidocaine.

3. The effect of shear stress on the human BBB co-culture model: morphology, surface charge and gene expression

Since the stem cell based human BBB co-culture model was only investigated under static conditions, we also aimed to test the effect of fluid flow in the LOC device on barrier integrity, surface charge and ESG of this BLEC/pericyte model using both functional and transcriptomic assays. We could demonstrate that flow conditions increased barrier properties and changed EC morphology (Figure 8).

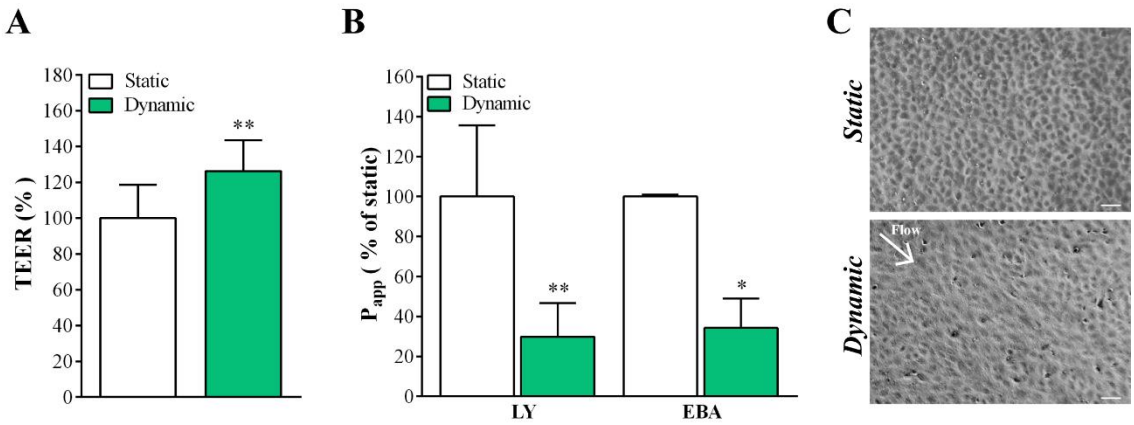

Figure 8. Characterization of the human BBB model in the lab-on-a-chip (LOC). (A) TEER results were normalized to the values of the static condition which did not receive any fluid flow (n=12). (B) Apparent permeability coefficient (P_{app}) of the human BBB model under static and dynamic conditions for Lucifer yellow (LY) and Evans blue labeled albumin (EBA) marker molecules. Data is shown as the % of the static condition (n=2-4). (C) Phase contrast images of brain endothelial cells under static and dynamic conditions. Scale bar: 100 µm. Values are presented as means ± SD, Unpaired t-test, *p<0.05, **p<0.01.

Flow also induced several key general endothelial and BBB-related genes in brain ECs. The effect on the surface glycocalyx was corroborated by the gene enrichment profiles (Figure 9). The most significantly upregulated pathways after the introduction of flow conditions were the extracellular matrix and structure pathways and the ESG-related pathways.

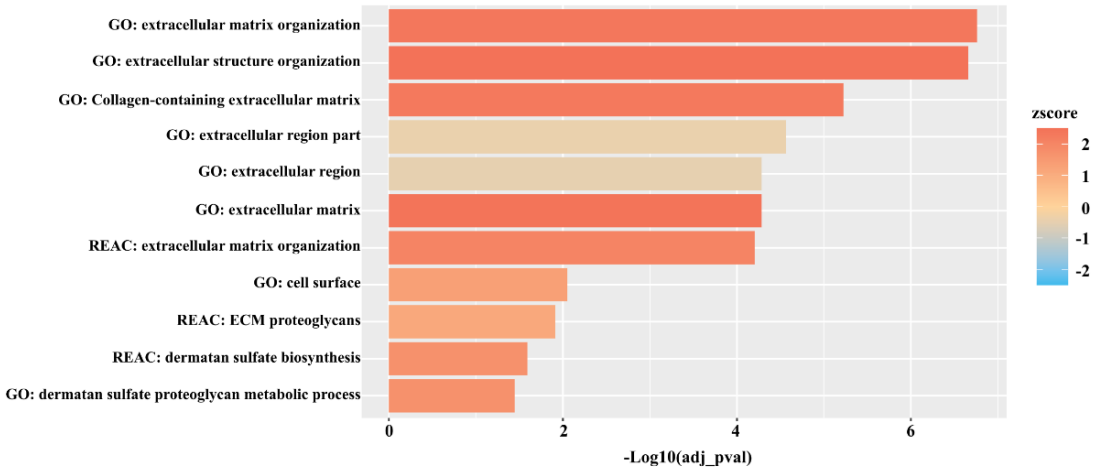

Figure 9. Functional profiling analysis of extracellular matrix-related pathways in static versus dynamic condition. The x-axis represents the statistical significance calculated using g:Profiler while the z-score represents the tendency of the regulation of these pathways calculated using GO plot.

Flow conditions not only upregulated extracellular matrix and glycocalyx-related genes and pathways but turned the brain endothelial cell surface more negatively charged and richer in lectin binding sites (Figure 10).

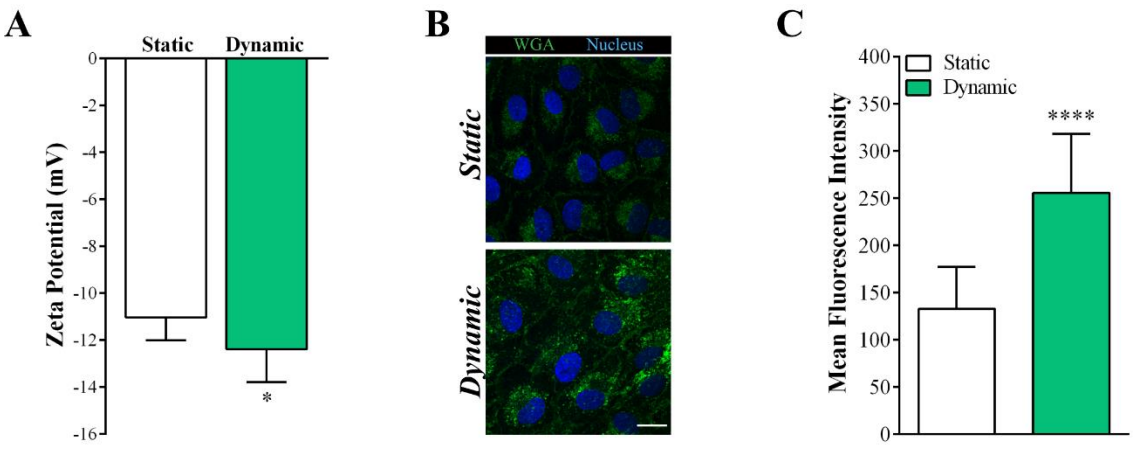

Figure 10. (A) Zeta potential measured by laser Doppler velocimetry (means ± SD, n=10; unpaired t-test, * $p<0.05$ compared to static condition). (B-C) Staining of ESG on brain endothelial cells with fluorescently labelled wheat germ agglutinin (WGA) lectin. Scale bar: 20 µm. Image analysis values are presented as means ± SD, n=72; unpaired t-test, ****$p<0.0001$ compared to static condition.

Conclusion

Based on our investigations we found the surface charge of rat and human brain ECs from three different BBB models highly negative, supporting a previous observation on bovine brain ECs. Moreover, we are the first to describe the modulation of the brain surface charge using neuraminidase enzyme and the cationic lipophilic drug lidocaine and how that changes the barrier properties of different BBB models. Our data draw attention to the importance of the ESG as a physical barrier of the BBB and to the interaction of charged drugs at the level of BBB. The results obtained by the use of our LOC device strengthen the significance of using flow in BBB models. The human co-culture BBB model can be a novel tool to study the role of cell surface glycocalyx in BBB physiology and pathology. The experimental data of our studies contribute to the knowledge about the surface charge and glycocalyx of brain ECs and the interaction of charged molecules at the BBB, and highlight the importance of fluid flow generated shear stress in BBB modeling.

List of publications

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Cumulative impact factor: 19.21

Publications related to the dissertation

I. **Santa-Maria AR**, Walter FR, Valkai S, Brás AR, Mészáros M, Kincses A, Klepe A, Gaspar D, Castanho MARB, Zimányi L, Dér A, Deli MA.
Lidocaine turns the surface charge of biological membranes more positive and changes the permeability of blood-brain barrier culture models.
Biochimica et Biophysica Acta - Biomembranes 1861: 1579-1591. (2019)
IF: 3.411

II. Kincses A, **Santa-Maria AR**, Walter FR, Dér L, Horányi N, Lipka DV, Valkai S, Deli MA, Dér A.
A chip device to determine surface charge properties of confluent cell monolayers by measuring streaming potential.
Lab on a Chip 20: 3792-3805. (2020)
IF: 6.774

III. **Santa-Maria AR***, Heymans M*, Walter FR, Culot M, Gosselet F, Deli MA, Neuhaus W.
Transport Studies Using Blood-Brain Barrier in Vitro Models: A Critical Review and Guidelines.
Handbook of Experimental Pharmacology doi: 10.1007/164_2020_394 (2020)
IF: -
**Authors contributed equally to the publication*

IV. **Santa-Maria AR**, Walter FR, Figueiredo R, Kincses A, Vigh J, Heymans M, Culot M, Winter P, Gosselet F, Dér A, Deli MA
Flow induces glycocalyx-related genes and negative surface charge in a lab-on-a-chip human blood-brain barrier model.
Manuscript in preparation

Other publications not related to the dissertation:

I. Gróf I, Bocsik A, Harazin A, **Santa-Maria AR**, Vizsnyiczai G, Barna L, Kiss L, Fűr G, Rakonczay Jr Z, Ambrus R, Szabó-Révész P, Gosselet F, Jaikumpun P, Szabó H, Zsembery Á, Deli MA.
The Effect of Sodium Bicarbonate, a Beneficial Adjuvant Molecule in Cystic Fibrosis, on Bronchial Epithelial Cells Expressing a Wild-Type or Mutant CFTR Channel.
International Journal of Molecular Sciences 21: 4024. (2020)
IF: 4.556

II. Francisco DMF, Marchetti L, Rodríguez-Lorenzo S, Frías-Anaya E, Figueiredo RM, **BtRAIN Network**, Winter P, Romero IA, de Vries HE, Engelhardt B, Bruggmann R.
Advancing brain barriers RNA sequencing: guidelines from experimental design to publication.
Fluids Barriers of the CNS 17:51. (2020)
IF: 4.470

III. Kanyó N, Kovács K, Saftics A, Székács I, Péter B, **Santa-Maria AR**, Walter FR, Dér A, Deli MA, Horváth R.
Glycocalyx regulates the strength and kinetics of cancer cell adhesion: biophysical models based on high resolution label-free optical data.
Scientific Reports, under revision
IF: 3.998

IV. Walter FR, **Santa-Maria AR**, Mészáros M, Veszelka S, Dér A, Deli MA
Surface charge, glycocalyx and blood-brain barrier function *(review)*
Tissue Barriers, under submission
IF: -

Declaration

As the corresponding author, I declare that the author Ana Raquel Pato Santa Maria contributed significantly to the results of the scientific publications listed below. I attest that the results presented in this thesis were not presented in any other PhD thesis.

I. **Santa-Maria AR**, Walter FR, Valkai S, Brás AR, Mészáros M, Kincses A, Klepe A, Gaspar D, Castanho MARB, Zimányi L, Dér A, Deli MA. Lidocaine turns the surface charge of biological membranes more positive and changes the permeability of blood-brain barrier culture models. *Biochimica et Biophysica Acta - Biomembranes* 1861: 1579-1591. (2019)

II. Kincses A, **Santa-Maria AR**, Walter FR, Dér L, Horányi N, Lipka DV, Valkai S, Deli MA, Dér A. A chip device to determine surface charge properties of confluent cell monolayers by measuring streaming potential. *Lab on a Chip* 20: 3792-3805. (2020)

III. **Santa-Maria AR***, Heymans M*, Walter FR, Culot M, Gosselet F, Deli MA, Neuhaus W. Transport Studies Using Blood-Brain Barrier in Vitro Models: A Critical Review and Guidelines. *Handbook of Experimental Pharmacology* (2020)

IV. **Santa-Maria AR**, Walter FR, Figueiredo R, Kincses A, Vigh J, Heymans M, Culot M, Winter P, Gosselet F, Dér A, Deli MA Flow induces glycocalyx-related genes and negative surface charge in a lab-on-a-chip human blood-brain barrier model. Manuscript in preparation

Szeged, 14th December 2020

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