Human iPSC-based models of the CNS: attaining cellular biofidelity through conventional and advanced culture systems

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Academic Dissertation which, with due permission of the KTH Royal Institute of Technology, is submitted for public defence for the Degree of Doctor of Philosophy on Wednesday the 15th June 2022, at 10:00 a.m. in D2, Lindstedtsvägen 9, Stockholm

Doctoral Thesis in Biotechnology
KTH Royal Institute of Technology
Stockholm, Sweden 2022

ISBN 978-91-8040-280-4
TRITA-CBH-FOU-2022:37

Printed by: Universitetsservice US-AB, Sweden 2022

Cover illustration: Human induced pluripotent stem cell-derived astrocytes expressing the astrocyte-specific S100 calcium binding protein B (green).

ABSTRACT

Brain development is a highly orchestrated process that entails changes in microenvironmental cues and growth factor gradients, which set the tempo for proper development of the rudimentary structures of the brain and the generation of neurons, astrocytes, and oligodendrocytes. Another intricate feature of the brain is the blood-brain barrier (BBB). The BBB consists of specialized endothelial cells that form a semipermeable barrier between the blood and the brain; hence, the BBB plays an important part in protecting the brain from blood-borne pathogens. *In vitro* modeling is inherently limiting, an artificial microenvironment that is usually not in tune with *in vivo* conditions. Thus, understanding these cues and growth factor conditions is pivotal for proper *in vitro* modeling and achieving cell biomimicry in vitro. Stem cell differentiation is highly amenable to growth factors and microenvironmental cues that can alter the expression of proteins. Advanced *in vitro* culturing considers microenvironmental cues and applies a more holistic aspect to *in vitro* modeling. This thesis evaluates microenvironmental cues in neural stem cell generation and astrocyte generation by employing induced pluripotent stem cells (iPSC). This thesis introduces a new protocol for generating human iPSC-derived astrocytes in under 28 days. By creating an astrocytogenic milieu, neural stem cells give rise to star-shaped astrocytes that encompass many traits previously unmet in iPSC-derived astrocytes, namely, ICAM-1 expression under inflammatory stimulation, glutathione synthesis and secretion. A follow-up study in this thesis presents a proteomic analysis between primary fetal astrocytes and iPSC-derived astrocytes. Microphysiological systems impart a more appropriate culturing microenvironment and influence cell fate and functionality. Another study of this thesis focuses on the differences between conventional and microphysiological culture systems in iPSC reprogramming and the generation of neural stem cells. Lastly, *in vitro* modeling of the blood-brain barrier (BBB) is also investigated. Specifically, 1) a human iPSC-BBB-like model is used to evaluate the permeability of a drug delivery system based on nanostructured lipid carriers and 2) a vessel-like structure with a 3D glioma model.

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SAMMANFATTNING

Hjärnutveckling är en mycket orkestrerad process som medför förändringar i mikromiljömässiga signaler och tillväxtfaktorgradienter, som sätter tempot för korrekt utveckling av hjärnans rudimentära strukturer och generering av neuroner, astrocyter och oligodendrocyter. Blod-hjärnbarriären (BBB) bildas av specialiserade endotelceller som bildar en semipermeabel barriär mellan blodet och hjärnan. Därför spelar BBB en viktig roll för att skydda hjärnan från blodburna patogener In vitro-modellering är i sig begränsande, en artificiell mikromiljö som vanligtvis inte är i samklang med *in vivo*-förhållanden. Därför är förståelse av dessa signaler och tillväxtfaktorförhållanden avgörande för korrekt in vitro-modellering och för att uppnå cellbiomimik in vitro. Stamcellsdifferentiering är mycket mottaglig för tillväxtfaktorer och mikromiljömässiga signaler som kan förändra uttrycket av proteiner. Avancerad in vitro-odling tar hänsyn till mikromiljömässiga signaler och tillämpar en holistisk syn på in vitro-modellering. Denna avhandling syftar till att utvärdera mikromiljömässiga signaler i generering av neurala stamceller och generering av astrocyter genom att använda inducerade pluripotenta stamceller (iPSC). Denna avhandling introducerar ett nytt protokoll för att generera mänskliga iPSC-härledda astrocyter på under 28 dagar. Genom att skapa en astrocytogen miljö ger neurala stamceller upphov till stjärnformade astrocyter som omfattar många egenskaper som tidigare inte uppfyllts i iPSC-härledda astrocyter, nämligen ICAM-1-uttryck under inflammatorisk stimulering, glutationsyntes och sekretion. En uppföljningsstudie i denna avhandling presenterar en proteomisk analys mellan primära fetala astrocyter och iPSC-härledda astrocyter. Mikrofysiologiska system ger en mer lämplig odlingsmikromiljö och påverkar cellens öde och funktionalitet. En annan studie av denna avhandling fokuserar på skillnaderna mellan konventionella-och mikrofysiologiska odlingssystem i iPSC-omprogrammering och generering av neurala stamceller. Slutligen undersöks också in vitro-modellering av blod-hjärnbarriären (BBB). Specifikt används 1) en mänsklig iPSC-BBB-liknande modell för att utvärdera genomträngligheten hos ett läkemedelsleveranssystem baserat på nanostrukturerade lipidbärare och 2) en kärlliknande struktur med en 3D-gliommodell.

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Contents

List of Publications

This thesis is based on the following peer-reviewed journals and manuscripts.

Paper I Generation of human iPSC-derived astrocytes with a mature star-shaped phenotype for CNS modeling.

D.Voulgaris, P. Nikolakopoulou, A. Herland, *Stem Cell Reviews & Reports*, 2022.

Paper II Proteomics analysis of iPSC-derived astrocytes through high throughput thermal proteome profiling.

D. Voulgaris, J. Rogal*, P. Nikolakopoulou*, A. Saei Dibavar, M. Gaetani, A. Herland, Manuscript.

Paper III On-chip neural induction boosts neural stem cell commitment and stabilization: towards a pipeline for hiPSC-based therapies.

D. Voulgaris*, S. Jain*, R. Hesen, M. Moslem, A. Falk, A. Herland, Manuscript.

Paper IV Dual effect of TAT functionalized DHAH lipid nanoparticles with neurotrophic factors in human BBB and microglia cultures.

S. Hernando*, P. Nikolakopoulou*, **D. Voulgaris**, R. M. Hernandez, M. Igartua, A. Herland, *Fluids and Barriers of the CNS, 2022.*

Paper V Three Dimensional Microvascularized Tissue Models by Laser-Based Cavitation Molding of Collagen.

A. Enrico, **D. Voulgaris**, R. Östmans, N. Sundaravadivel, L. Moutaux, A. Cordier, F. Niklaus, A Herland, G. Stemme, *Advanced Materials, 2022.*

*Equal contribution

The contribution of Dimitrios Voulgaris to each publication and manuscript listed above, major (●●●), partial (●●), minor (●).

Paper	Experiment	Analysis / Evaluation	Writing
I	●●●	●●●	●●●
II	●●●	●●●	●●
III	●●●	●●●	●●●
IV	●●	●●	●
V	●	●	●●

Additionally, the author has contributed to the following peer-reviewed papers and manuscripts.

Paper VI Continuous Monitoring Reveals Protective Effects of N-Acetylcysteine Amide on an Isogenic Microphysiological Model of the Neurovascular Unit.

I. Matthiesen, **D. Voulgaris**, P. Nikolakopoulou, T. E Winkler, A. Herland, *Small*, 2021.

Paper VII Recent progress in translational engineered in vitro models of the central nervous system.

P. Nikolakopoulou*, R. Rauti*, **D. Voulgaris**, I. Shlomy, B. M Maoz, A. Herland, *Brain*, 2021.

Paper VIII Barrier properties and transcriptome expression in human iPSC-derived models of the blood–brain barrier.

L. Delsing, P. Dönnes, J. Sánchez, M. Clausen, **D. Voulgaris**, A. Falk, A. Herland, G. Brolén, H. Zetterberg, R. Hicks, J. Synnergren, *Stem Cell Reports*, 2018.

Paper IX A Human iPSC-derived Microphysiological Model for Blood-Brain Barrier Permeability Screening.

L. Delsing, B. Ulfenborg, **D. Voulgaris**, H. Zetterberg, A. Herland, R. Hicks, J. Synnergren, Manuscript.

Acronyms

ALS: Amyotrophic lateral sclerosis
BBB: Blood-brain barrier
BMEC: Brain microvasculature endothelial cell
BMP: Bone morphogenetic protein
CNS: Central Nervous System
ECM: Extracellular matrix
ESC: Embryonic stem cells
FGF: Fibroblast growth factor
GFAP: Glial fibrillary acidic protein
GW: Gestational week
HFA: Human fetal astrocytes
HUVEC: Human umbilical cord endothelial cell
iPSC: Included pluripotent stem cells
MPS: Microphysiological systems
NSC: Neural stem cell
PDMS: Polydimethylsiloxane
PLO: Poly-L-ornithine
SHH: Sonic hedgehog
SMAD: Small mothers against decapentaplegic
TEER: Transendothelial electrical resistance
WNT: Wingless-related integration site

Aims & Structure

This thesis and papers aim to improve *in vitro* models through a holistic view of *in vitro* traditional and advanced culture systems. *In vitro* culture systems usually lack many *in vivo* components. Such components are, among others, interactions with other cell types, interaction with the extracellular matrix, a 3D structure and turnover of cellular cues. Specifically, I focused on creating advanced *in vitro* models of the Central Nervous System (CNS) by capitalizing on induced pluripotent stem cell (iPSC) models. Advanced *in vitro* modeling attempts to bring *in vitro* models closer to *in vivo* conditions. Stem cell differentiations are highly instructed by culturing conditions such as availability of growth factors, cell-to-cell signaling and cell-to-extracellular matrix (ECM) signaling.

The aim of this thesis includes 1) development of a novel astrocytic differentiation protocol from neural stem cells (NSC), 2) iPSC reprogramming and NSC generation and characterization of these processes in two different culturing formats, and 3) *in vitro* blood-brain barrier (BBB) modeling and characterization; the BBB was modeled either via iPSC differentiation or through primary cells.

In paper I, I developed an astrocytic protocol based on a primary astrocytic media, an astrocytic tuned extracellular matrix (denatured collagen, gelatin) and proper seeding density that regulate cell-to-cell signaling. A proteomic analysis was employed to further characterize hiAstrocytes in paper II. In paper III, the effects of cell-to-cell signaling were explored in the context of iPSC reprogramming and NSC generation (neural induction). Cell-to-cell signaling was indirectly geared by comparing traditional (well plates) cultures with microphysiological system cultures. In papers IV and V, the focus was on recreating some elements of the BBB. More specifically, paper IV focuses on evaluating the permeability of nanostructured lipid carriers through an iPSC barrier that has *in vivo*-like transendothelial electrical resistance (TEER). Paper V establishes a 3D platform using umbilical cord endothelial cells and 3D glioma culture, creating the first steps towards a vascularized brain tumor model from a biological perspective.

To put these aims and papers in context, this thesis starts with a brief introduction to brain development, describing the different developmental stages and cell types. Next, I elaborate on the state-of-the-art CNS *in vitro* models in Chapter 2, and Chapter 3 focuses on microenvironmental cues and microphysiological systems (MPS) and how they can increase the *in vivo* fidelity of *in vitro* models. Finally, in Chapter 4, I summarize the conclusions of this thesis and give an outlook on the future development of this field in Chapter 5.

Chapter 1
Brain development

1.1 Embryogenesis

Following the formation of a zygote from the oocyte and spermatozoon, the totipotent zygote ultimately gives rise to a hollow ball structure containing the inner cell mass, the blastocyst cavity (blastocoele), and the outer cell layer (trophoblast), (Fig. 1). The inner cell mass consists of pluripotent stem cells, namely embryonic stem cells (ESC). ESCs have been extensively used in research and can proliferate virtually indefinitely *in vitro* in the presence of growth factors and hormones that retain their stemness and self-renewal. Such proteins are basic fibroblast growth factors (FGF), transforming growth factor-beta, insulin, transferrin and sodium selenite [1].

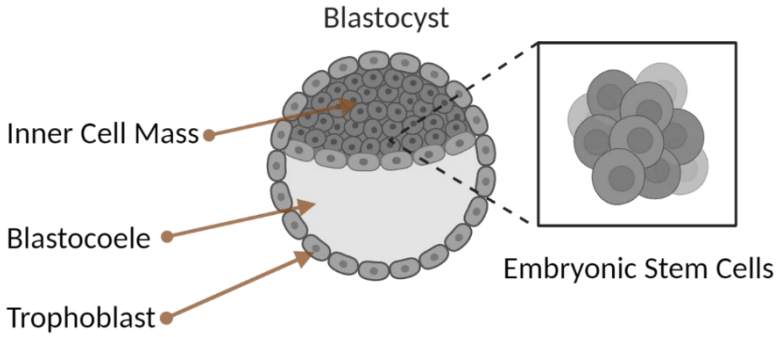

Figure 1 Graphical representation of the blastocyst, a cellular structure generated around 5 days post-conception. The blastocyst contains the inner cell mass, blastocoele and trophoblast. The inner cell mas represent, *in vitro*, the embryonic stem cells, which can give rise to all 3 germ layers. The trophoblast gives rise to the extraembryonic tissue. Created with BioRender.com.

The inner cell mass, through the process of gastrulation, gives rise during the gestational week (GW) 2 to the 3 germ layers: the ectoderm, mesoderm, and endoderm. The endoderm gives rise to internal organs such as the liver and pancreas, while the mesoderm gives rise to structures such as cartilage, bone, blood, and blood vessels. Lastly, the ectoderm gives rise to the epidermal ectoderm, which generates skin and nails and the neuroectoderm, which generates the CNS.

With the advent of iPSC [2], access to these embryonic stem cell-like cells greatly increased the number of stem cell differentiation protocols. Pluripotency is the capacity of a cellular entity to self-propagate and the capacity, upon withdrawal of growth factors, to generate all germ layers, similarly to the process of gastrulation of the inner cell mass (i.e., ESCs). IPSCs or ESCs can further be restricted (developmentally) to multipotent stem cells, such as NSC. Multipotent stem cells are non-terminally differentiated cells that retain their self-replicating machinery. However, multipotent stem cells are germ layer-restricted and cannot produce cells of other lineages.

1.2 The neuroectoderm fate

Brain development starts at GW3; during GW3, a mesoderm-derived structure, the notochord, secretes bone morphogenetic protein (BMP) inhibitors, such as noggin, chordin and follistatin [3,4]. The ectoderm in the surrounding vicinity of the secretion of BMP inhibitors from the notochord acquires a neuroectoderm cellular fate. In contrast, BMP pathway activation in the rest of the ectoderm generates the epidermal ectoderm. Through the neurulation process, the neural plate elongates its folds and closes, making the first well-defined structure of the CNS, the neural tube. BMP signaling dominates the brain's development temporally. Initially, BMP signaling is inhibited to allow neurulation of the ectoderm; at later stages, BMP signaling [5] and Sonic Hedgehog (SHH) [6] define the dorsoventral polarization of the neural tube. This highly regulated patterning process results from the synergistic action of opposing gradients, also known as the French flag model of morphogenesis.

The French flag model of morphogenesis was proposed by Lewis Wolpert in 1969 [7] and proposes that during development, cells receive a positional identity through gradients emanating from cells dictating cellular fate and positional identity (i.e., organizers). These organizers secrete factors that affect cellular fates based on the proximity of the cell to the organizers (Fig. 2).

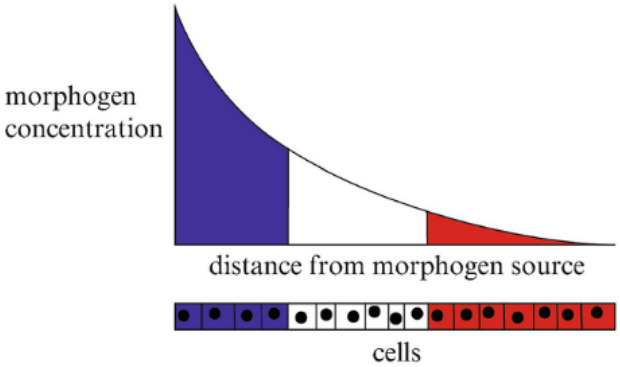

Figure 2 The French flag model of morphogenesis. The same morphogen secreted by an organizer differentially affects cellular fate (e.g.," blue", "white" or "red") based on the concentration levels of the morphogen, which are regulated by the distance of the cells from the organizer. Cells near the source of the morphogen acquire a "blue" cellular fate, while cells spaced further apart acquire "white" and "red" cellular fates. Reprinted with permission from [8].

Based on knowledge of pathways during brain development, many neural induction protocols (i.e., the generation of NSCs from iPSCs or ESCs) have been used in literature and applied to iPSCs and ESCs. Therefore, different names are attributed to cellular entities that correspond to the different cellular structures during brain development, as reviewed in Conti and Cattaneo, 2010 [9]. However, neural stem cells and neuroepithelial cells are used interchangeably in this thesis and articles, and there is no association of these terms to different structures such as the neural plate or, the more regionally patterned brain structure, the neural tube.

1.2.1 Neural stem cells

NSCs are multipotent stem cells that line the neural tube and can generate neurons, astrocytes, and oligodendrocytes. The neural tube gives rise to the primary vesicles, the forebrain, midbrain, and hindbrain. These rudimentary structures hold neural stem cells with a positional identity analogous to the residing structure. Hence, NSCs are a heterogeneous population of temporally and positionally distinct cells. This heterogeneity is a crucial understanding of the concept of NSCs and why perhaps they are so many terms attributed to the stem cells of the neuroectoderm.

1.2.2 The history, development, and functionality of astrocytes

Rudolf Virchow first identified astrocytes in 1858 [10]. They were labeled the "non-neuronal" part of the brain, the glue (hence, the glia), a non-cellular entity that holds everything together. Astrocytes gained their current name by Michael von Lenhosék in 1893. Developmentally, neurons precede astrocytes during development (Fig. 3). The positional identity of neural stem cells across the neural tube imparts specific functionalities and identity to the generated progeny. Specifically, neurons derived from the dorsal side of the neural tube give rise to motor neurons. Adhering to the same paradigm, astrocytes also have a positional identity [12].

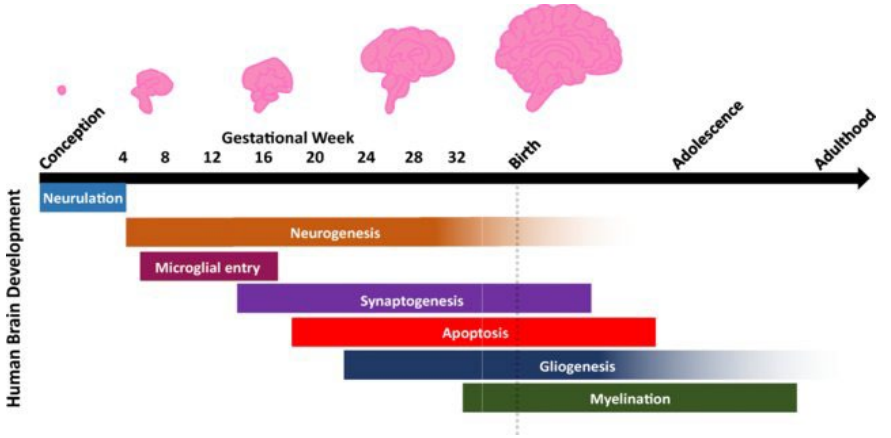

Figure 3 Timeline of brain development. Neurulation is followed by neurogenesis around GW4. Shortly after, microglia invasion occurs. Gliogenesis follows last and persists postnatally. Reprinted with permission from [11].

The switch from neurogenesis to astrogenesis is suggested to occur when astrogenesis-inhibiting factors are no longer exerted on neural stem cells. Specifically, the JAK/STAT pathway is a master regulator of astrogenesis, and it is initially hampered by neurogenic factors [13]. Astrogenesis starts around GW18 and persists postnatally.

An ever-growing body of research supports that astrocytes are more than just supporting cells. Astrocytes serve a plethora of functions in the CNS, from brain homeostasis regulators to key neuroinflammation players; these star-shaped cells are truly multitaskers of the brain. Their interaction with neurons is crucial for brain homeostasis. Astrocytes regulate synaptic transmission [14,15], the so-called tripartite synapses. Additionally, astrocytes take up excess extracellular glutamate [16–18] at the synaptic cleft. Most importantly, neurons cannot synthesize antioxidants *de novo* and depend on astrocytes to provide precursor molecules [19]. Hence, neurons are highly dependent on astrocytes for proper function.

As part of their homeostasis repertoire, astrocytes are directly involved in the glymphatic system [20], a system for brain waste removal. The waste clearance capabilities of astrocytes do not stop at glutamate clearance; astrocytes, through the expression of aquaporin 4 in their end-feet, are the major component of the glymphatic system. Maiken Nedergaard in 2013, coined the term "glymphatic system" as a token of recognition of astrocytes' involvement in the waste removal of the brain.

Astrocytes are also involved in the brain's innate immune response; they envelop with their astrocytic end-feet the majority of brain blood vessels and are the first cell type to respond to blood-derived insults [21]. Additionally, they secrete soluble mediators such as interleukin-6 [22], interleukin-8 and monocyte chemoattractant protein-1 [23] upon inflammation.

1.3 Challenging the "neuro-centric" archetype and the rise of the stars

Neuroscientists have extensively studied neurons in various neurodegenerative and neurodevelopmental contexts. Alexander disease is perhaps one of the first diseases identified to be caused exclusively by astrocytes [24]. Alexander disease is a leukodystrophy where clinical symptoms, among others, include macrocephaly and seizures. Mutations in an astrocytic-exclusive (among the cells of the CNS) protein, glial fibrillary acidic protein (GFAP), are the cause of Alexander disease. Astrocytes directly contribute to disease progression via a positive feedback loop with T cells in multiple sclerosis. This so-called "two waves' theory" proposes that cytokines secreted by activated T cells activate astrocytes; consequently, activated astrocytes secrete chemoattract proteins that further recruit T cells [25–27]. Astrocytes are also affected by other organs, such as the liver. Liver failure leads to ammonia building up in the blood and crossing the BBB. Astrocytes use ammonia to convert glutamate to glutamine; hence the excess ammonia in the brain causes astrocytes to convert even more glutamate to glutamine; astrocytes eventually swell [28], and there is also a loss of one of the major glutamate clearance transporters' expression, the excitatory amino acid transporter 2 [29]. Hepatic encephalopathy can be fatal if left untreated.

A notable neurodegenerative disease is amyotrophic lateral sclerosis (ALS), affecting motor neurons. During the disease progression of ALS, motor neurons are gradually dying, causing a loss of muscle control. The scientific community focused on how mutations affect neuronal function and health [30,31], neglecting the impact of astrocytes on these neuronal populations. Indeed, Arredondo et al. in March 2022, unveiled astrocytes' direct involvement in ALS [32]. Astrocytes from ALS patients showed increased secretion levels of inorganic polyphosphate that killed motor neurons. That study is just one example of how moving away from neuroscience's "neuro-centric" doctrine, and combining iPSC technology, can unveil etiologies and better understand neurodegenerative diseases.

Researchers are slowly moving away from the "neuro-centric" idea of studying neuroscience and moving forwards to understand CNS pathologies holistically and from the side of astrocytes. Ben A. Barres was one of the researchers that laid the contemporary foundations to understand better astrocyte biology, heterogeneity and function [33].

1.4 The blood-brain barrier

The BBB is an intrinsic characteristic of the brain, and it serves as a gatekeeper to the brain parenchyma. The BBB is a collection of cells that form a semipermeable barrier that allows only passive diffusion of lipophilic molecules (e.g., O2, hormones) and small non-polar molecules. Additionally, macromolecules necessary for the brain are actively transported through specialized transporters of the BBB (e.g., glucose). The core cellular components of the BBB are brain endothelial cells, astrocytes, and pericytes. This fortified barrier prevents blood-borne infections while at the same time also preventing therapeutics from entering the brain. Brain regionality also affects BBB functionality; for example, P-glycoprotein expression is higher in the white matter than in the grey matter [34].

Furthermore, the BBB does not cover the whole brain. Specifically, the BBB is lacking from circumventricular organs such as the pituitary gland and choroid plexus. Importantly, CNS diseases lead to the breakdown of the barrier, which has detrimental effects on disease progression.

Unlike endothelial cells of the rest of the body, brain endothelial cells are non-fenestrated and have continuous tight junctions (Fig. 4) [35]. In addition, recent findings point to astrocytes having a pivotal role in BBB development and maintenance [36]; however, their role in development is still contested.

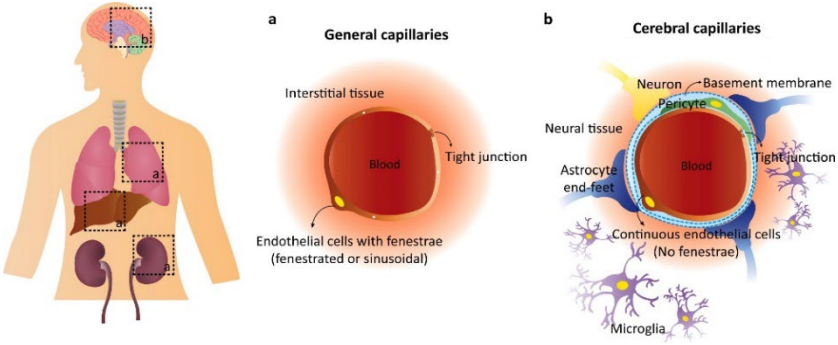

Figure 4 Differences between (a) general and (b) cerebral capillaries. General capillaries are fenestrated or sinusoidal, and transport is mainly done via fluid-phase or endocytosis (receptor-mediated). Cerebral capillaries (brain endothelial cells) are non-fenestrated and continuous. Passive transport is limited to small lipophilic molecules. Larger molecules are actively transported through specialized transport systems. Reprinted with permission from [37].

Nevertheless, researchers have shown that astrocytes induce BBB characteristics in non-BBB endothelial cells *in vivo* [38]. Astrocytes not only secrete growth factors that regulate the tightness of the BBB [38] but additionally, 90% of the brain capillaries are covered with astrocytic processes. Astrocytes also regulate blood flow [39]. The intimate relationship between astrocytes and brain endothelial cells has been exemplified in numerous studies [35,40–44].

1.5 Microglia

Microglia are the specialized immune cells of the brain. Originating from a mesoderm lineage, microglia are postulated to invade the brain around GW4 as hematopoietic precursors. After brain entry, they are terminally differentiated into the brain-specific immune cells called microglia. Even though the term" glia" is used, developmentally, they do not share characteristics with the glial cells, such as astrocytes and oligodendrocytes. The cross-talk between astrocytes and microglia is an important regulator of brain homeostasis [45–47].

The interplay between astrocytes and microglia extends to pathological conditions. More specifically, in pathological conditions, microglia and astrocytes are truly partners in crime; LPS-activated microglia have been shown to release complement component C1q and cytokines such as TNF, which in turn activate astrocytes. Consequently, astrocytes no longer support neurons that ultimately die [48]. Another example of cross-talk between those two cell types is the IL-3-mediated arming of microglia. Microglia are motile cells, continuously scavenging and probing in the brain; upon the recognition of Aβ plaques, microglia upregulate IL-3Ra. Astrocytes continuously secrete IL-3, which binds to its receptor on microglia bestowing microglia with extra motility and an acute immune response [49].

Chapter 2

CNS in vitro models

In vitro studies of the CNS have been historically done through primary cells or cell lines, and in the last decade, iPSC-derived CNS models have gained momentum. iPSC models are frequently advantageous over primary models in terms of costs, availability and, in some cases, cellular functionality. Furthermore*, in vivo* work has vested the scientific community with cues and insights into developmental aspects. This knowledge has been the basis for the majority of differentiation protocols to emulate the developmental process *in vitro* through the introduction of ECM or appropriate growth factors. Currently, animal studies are an irreplaceable step in the drug discovery process. Nevertheless, in this thesis, comparisons between *in vivo* and *in vitro* will be made only in the context of *in vivo* functionality. Therefore, a comparison of *in vitro* models (primary or iPSCs) with animal work will not be discussed and lies outside the scope of this thesis.

2.1 Primary models and cell lines

Primary human astrocytes are possibly the cell source with the greatest *in vivo* proximity. Isolation of astrocytes can either be done from adult post-mortem human tissues or aborted fetuses. Isolation from aborted fetuses is carried out between GW 19-21. Human fetal astrocytes (HFA) retain most of the host tissue functionalities. Additionally, immortalized astrocytic lines have also been used. However, immortalized astrocytic lines, even though positive for astrocytic markers, severely underperform (compared to HFA) in astrocyte-associated functionalities such as glutamate clearance and glutathione synthesis [50], limiting their usefulness.

Cells for modeling the BBB have been used, among others: primary microvasculature endothelial cells (BMECs), cell lines such as hCMEC/D3, and human umbilical cord endothelial cells (HUVECs). The ease of access and cost-friendly nature of HUVECs make this cell source an ideal primitive model of endothelial nature even though the brain endothelial characteristics are lacking. Another barrier model that has been used in research is the Caco-2. Caco-2 cells are epithelial; nevertheless, they exhibit barrier properties that render them useful as *in vitro* barrier models.

2.2 iPSC models

IPSC models have revolutionized our understanding of developmental processes. iPSCs are more accessible than embryonic stem cells, and therefore, many research groups have capitalized on the accessibility and generated iPSC models of the CNS. IPSCs can be reprogrammed from somatic cells, namely fibroblasts, urine (squamous) cells or peripheral blood mononuclear cells. IPSC reprogramming forces genes' expression, which are master regulators of pluripotency, specifically *OCT4*, *SOX2* and *NANOG*. They are numerous techniques for IPSC reprogramming through integrating (Lentivirus), non-integrating viruses (Sendai virus or adenovirus)[51], or mRNA[52].

Reprogramming techniques depend on the downstream process; for example, Sendai virus reprogramming is most suitable for solely translational applications since the virus vector does not enter the nucleus and can be diluted out after some post-transfection at slightly higher standard culture temperatures (39ºC) [53,54]. Nevertheless, iPSC for translational and cell therapy applications should be reprogrammed through non-integrating non-viral methods, such as mRNA. Reprogramming through mRNA has lower efficiency (reprogramming of fibroblasts lower than 4%) but provides a non-viral foot-print-free reprogramming of iPSC.

2.2.1 Neural Stem Cells

The generation of NSCs is usually influenced by the extensive developmental knowledge from *in vivo* developmental studies. The most widely used protocols are often based on dual-small mothers against decapentaplegic (SMAD) inhibition [55] or even single SMAD inhibition [9,56]. Noggin or small molecule analogs are often used to block the BMP pathway during neural induction. Additionally, growth factors can be added during neural induction to impart specific regionality to the neural stem cells. The growth factors that have been used to pattern neural stem cells are, among others, FGF8, WNT, retinoic acid and SHH [57]. Depending on the protocol used, populations with limited proliferative capacity (neural precursors) or proliferative populations (NSCs) are generated. Neural precursors cannot be cultured as multipotent stem cells *in vitro* and terminally differentiate into neurons and glia, while proliferative NSC can be cultured as multipotent stem cells *in vitro* by adding mitogens.

Neural stem cells can be cultured *in vitro* in defined media in the presence of bFGF and are positive for the intermediate filament NESTIN; developmentally, they represent fetal rather than adult neural stem cells. NSCs generated through dual-SMAD inhibition with WNT activation can be propagated for over 100 passages under serum-free conditions. However, although they are stable in terms of proliferation, neural stem cells experience a transcriptomic shift with passaging that

could be attributed to the *in vitro* culture conditions and/or an internal circadian clock [58].

Of note, the gliogenic potential of NSCs is variable. Spontaneously differentiating NSC cells give rise to mainly neurons (70 - 90 %) [59,60], results in paper I also corroborate with that claim; growth factor withdrawal for 28 days led to the enrichment of neurogenic marker *DCX* and low expression of astrocytic markers (*CD44* and *S100B*). Alisch et al. in 2021 showed that NSCs generated from ESCs and iPSCs increase their gliogenic potential with successive cell division [61], recapitulating brain development where neurons are generated first, followed by astrocytes. This phenomenon was also explored in paper I, where higher passage NSCs, during astrocyte differentiation, enrich astrocytic genes compared to lower passage NSCs. The gliogenic potential of NSCs requires more thorough investigation to ascertain whether intrinsic or extrinsic factors confer gliogenic capacity to NSCs

2.2.2 Astrocytes

The generation of astrocytes from stem cells is an elaborate process, spanning 20 days to over 200 days (from the NSC stage). The variability of astrocytic differentiations is more complicated than the generation of NSCs, especially when differentiating cells from NSCs. The variability could be partly attributed to 1) the regionality of neural stem cells could potentially lead to discrepancies in protocols, and 2) the inherent heterogeneity of NSCs as described in Ch. 2.2.1.

That is also reflected in the number of published astrocytic differentiations [62]. The differentiation strategy of various protocols relies on media composition that recruits growth factors such as CNTF, bFGF, EGF, LIF or FBS. Notably, all reported monolayer differentiation protocols elected to use for ECM coating Matrigel or a combination of poly-L-ornithine (PLO) and laminin or fibronectin (Fig. 5). Interestingly, the PLO/laminin combination is used in neural stem cell culture and corresponds to the highest percentage of published papers on astrocyte differentiations, highlighting the lack of consideration of ECM when designing astrocytic protocols.

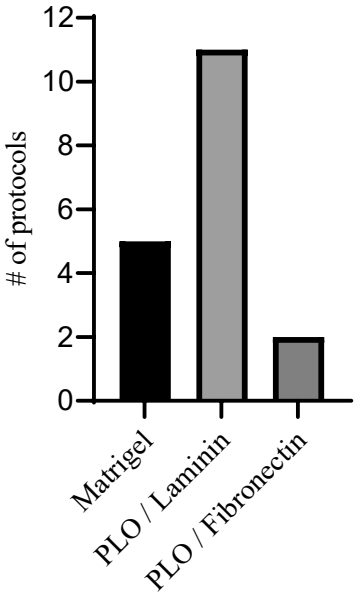

Figure 5 Summary of chosen ECM coating for various differentiation strategies, out of the 18 astrocytic monolayer differentiation protocols, 11 used a combination of PLO/Laminin, 5 used Matrigel and 2 used PLO / Fibronectin, data collected from [63–80].

Another important point of the PLO / Laminin coating is that it is tuned for neuronal differentiations [81]; hence, it could also be a remnant of the extensive research on promoting neuronal development *in vitro*. In paper I, we initially explored the impact of ECM coatings such as PLO / Laminin (data not shown), Matrigel, and a denatured form of collagen (gelatin) on the astrocytic commitment NSC.

2.2.3 Brain endothelial cells

The emergence of brain endothelial differentiation protocols that can, to some extent, recapitulate *in vitro* the *in vivo* characteristics of the brain endothelium paved the way for many drug transport studies [82–84]. These protocols are based on spontaneous differentiations of iPSCs followed by a retinoic acid treatment that increases the barrier's resistance to *in vivo* levels (>2000 $\Omega \times cm^2$). *In vivo* TEER has been measured in frogs and rats to be ~2000 $\Omega \times cm^2$ [85,86]. The barrier-like characteristics of this approach make this differentiation approach of BBB-like models ideal for transport studies. We explored the barrier properties of this hiPSC-brain endothelial-like protocol in paper IV, where we evaluated, among others, the permeability of this model with a drug delivery system (nanostructured lipid carrier).

2.3 Limitations of current primary and iPSC-derived CNS models

Primary BMECs cannot recreate any physiological relevant TEER (<200 Ω×cm^2), and drug transport studies are not relevant due to the leakiness. However, brain endothelial cells can be used for non-transport-related experiments, such as pulse-chase experiments. While posing a great tool for the *in vitro* assessment of transport studies, iPSC-BBB protocols fail to capture inflammatory responses [87]. Additionally, this specific differentiation strategy [82–84] generates a mixture of epithelial and endothelial types [88,89].

Adult astrocytes have limited functionality *in vitro* post isolation. Fetal astrocytes, while functional, are hard to access if biological variability needs to be tested. More importantly, fetal tissues cannot, by default, recapitulate *in vitro* any developmental disease, as opposed to iPSC reprogrammed from patients. Additionally, industry standards do not allow the use of fetal tissues rendering their usefulness strictly academic.

Even though cells isolated from primary tissues are the most relevant source of the tissues in question, a major hurdle that primary sources of cells entail is their de-differentiation that ensues following the isolation and expansion of these cells *in vitro.* For example, isolated brain endothelial cells have a more reactive phenotype once cultured [90]; consequently, it is undeniable that the isolation and *in vitro* expansion of these cell populations results in a transcriptomic shift that could potentially skew experimental data.

Fetal or adult tissue provides us with the exact biological age of the cells; for instance, fetal astrocytes can be harvested from aborted fetuses between GW19-22. A caveat with any iPSC product is the challenging task of determining the biological age of the resulting cells. NSCs are slightly easier to identify by comparing to fetal tissue. Additionally, the time window of the existence of fetal neural stem cells is rather small compared to astrocytes or brain endothelial cells. However, astrocytes and brain endothelial cells are harder to determine accurately; hence, the maturity of differentiated cells has always been debated. Differentiated cells are unlikely to resemble adult tissue since the adult tissues have undergone a developmental process that is challenging to recapitulate *in vitro*.

Additionally, there is a lack of astrocytic models after GW22 (excluding adult astrocytes). Hence, making it challenging to pinpoint the exact developmental age of astrocytic differentiations. Nevertheless, researchers have identified differentially enriched markers in fetal and mature astrocytes [91].

2.4 Cellular biofidelity

IPSC technology has undoubtedly enabled access to numerous differentiated cellular products for the research community. However, the differentiated cellular products occasionally fail to recapitulate the tissue of interest fully. For example, while capturing the *in vivo* resistance, brain endothelial-like cells fail to respond to inflammatory stimuli. The lack of cellular biofidelity could be attributed either to the protocol used itself or to microenvironmental cues, or lack thereof, that fail to generate functionalities akin to the tissue of interest. A good evaluation method of the cellular biofidelity of cellular products is *in vivo* grafting.

Chapter 3

Microenvironmental cues & microphysiological systems

Advanced *in vitro* culturing considers environmental cues and mechanical forces exerted *in vitro*. Advanced *in vitro* modeling includes, among others, 3D modeling, organoids and microphysiological systems (MPS); all these models attempt to recapitulate, to some degree, the complexity of the *in vivo* microenvironment. This chapter starts with the advantages of MPS *in vitro* modeling. Following that, advanced *in vitro* modeling and microenvironmental cues are discussed in the context of the BBB and astrocytes.

3.1 Microphysiological systems

MPS (or chips) permit a range of processes that cannot recreate in a conventional 2D well culture setting. MPS are typically the size of a cover slide (approximately 75 mm x 25 mm, Fig. 6) and are most frequently made of polydimethylsiloxane (PDMS), polymethylmethacrylate (PMMA) or cyclic olefin copolymer. PDMS is used in most MPS; the advantage of PDMS is that it is cost-efficient and more O2 permeable than the rest [92].

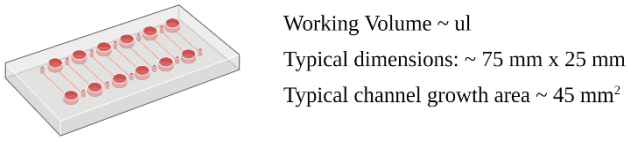

Figure 6 Graphical presentation of a typical chip design. Chips are often made from PDMS, and the typical dimensions are similar to a cover slide (75 mm x 25 mm). The channels typically hold media in the range of ul (~20-90 ul). The ratio of culture media over the growth area is smaller than in the conventional well plate. Created with BioRender.com.

MPS can be geared to accommodate a range of cell culture setups. From static MPS to the incorporation of flow, MPS are versatile tools for exploring elaborate biological processes. For example, spatial manipulation can provide a morphogen gradient model akin to the French flag morphogen model described in Ch. 2 (Fig. 2

and Fig. 7a). The importance of this feature comes into play during brain development; specifically, the neural plate, neural crest, and epidermis cellular fate are dictated by opposing gradients of FGF and WNT [93] (Fig 7b). Furthermore, morphogen gradients present *in vivo* can be recapitulated in MPS, while conventional culture systems lack spatial control over morphogens' concentration.

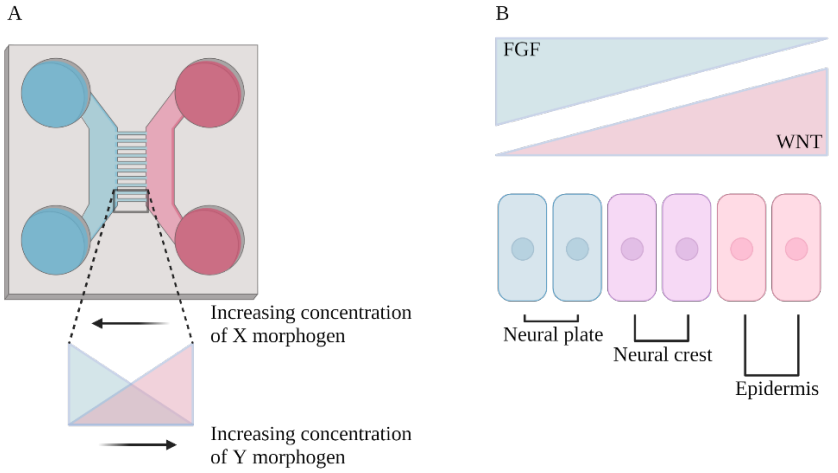

Figure 7 (a) Graphical presentation of a typical chip design. Chips are often made from PDMS, and the typical dimensions are like a cover slide (75 mm x 25 mm). Two different solutes are flowing through the chip (blue and red), creating an opposing gradient of the two different solutes (b) an example of opposing gradients during brain development, FGF and WNT, the various levels of the gradients give rise to the neural plate, neural crest, and epidermis. Created with BioRender.com.

MPS and modeling development have been a fertile ground for many studies; for example, researchers recreated the neural tube patterning on a chip [94,95]. In addition, the spatial control that MPS provides, combined with iPSC technology and disease models, can shed light on human-specific neurodevelopmental diseases.

Another commodity of MPS is the more physiological ratio of cell media volume over the growth area, potentially creating more *in vivo*-relevant *in vitro* models or even increasing biological processes. In MPS, endogenous growth factor secretion from cells is highly concentrated in chips instead of traditional culture systems, exemplified in paper 3. We showed that the MPS culture boosted neural development pathways during the establishment of neural stem cells compared to traditional well plate culture conditions.

Chips can also be linked together, akin to how organs are connected. Maoz et al. exemplified that by linking chips together, they could recapitulate the metabolic coupling between brain endothelial cells and neurons [96]. The complexity of this system can further be increased by multi-organ linked chips [97] that can provide insight into pharmacokinetics, drug distribution and potential side effects. The multi-organ chip system has the potential to provide human-specific readouts.

As mentioned earlier, flow is one of the key elements of MPS; one example is how a simulation of interstitial flow (0.1–0.3 uL/min) accelerated the generation of neurons from neural precursors that were seeded on a chip [98], further advantages of flow will be discussed in following sub-chapters.

3.2 Advanced in vitro modeling of the BBB

The brain endothelial cells in the capillaries are exposed to shear stress. This force that is exerted on endothelial cells is key to barrier characteristics. The BBB is an excellent example of where advanced *in vitro* culture can be applied to create an improved *in vitro* model. In addition, MPS, such as chips, enable the proper shear stress that these cells experience *in vitro*.

For example, Siddharthan et al. showed that when shear stress is applied to BMECs, the tight junction protein ZO-1 is upregulated [99]; additionally, it has been reported that shear stress increases the barrier's resistance [100]. In a more comprehensive evaluation of the impact of sheet stress on the BMECS, Cucullo et al. reported that shear stress is a key component of the BBB phenotype since they showed that the inclusion of shear stress resulted in enrichment of tight and adherens junctions [101]. Furthermore, they showed that the BBB functionality increased (upregulation of multidrug resistance transporters). Shear stress also affects the alignment of endothelial cells.

Another intricate element that separates the brain endothelium from non-CNS endothelium is that brain endothelial cells do not undergo a phenotypic transition from cobblestone to spindle-like [102,103]. , other reports suggest that BMECs transition to spindle-like morphology under flow conditions [104]. Regardless, endothelial cell response is more multifaceted than merely summed up to whether brain endothelial cells align or not to the direction of the flow. Specifically, the identity of the endothelium [105] (whereas arterial or venous) and the level of shear stress [106] should be added as important confounding variables when defining the endothelial cell's response to shear flow.

Geometry is another salient feature of the endothelium. Attempts at 3D renderings of the brain endothelium have been made using various techniques, such as viscous fingering [107], template rod [108–110] or more elaborate PDMS chips [111,112]. Nevertheless, 3D geometry, in the context of biological functionality of the BBB, has not been documented to provide an advantage over 2D conventional culture systems. Regardless, 3D rendering of the BBB provides an attempt for more physiological readouts, for instance, in paracellular permeability and pharmacokinetics.

3.3 Advanced in vitro modeling of astrocytes

There is a bi-directional influence between astrocytes and the microenvironment of other CNS cells. For example, the intimate relationship between astrocytes and brain endothelial cells has been explained previously; however, a noteworthy aspect is that astrocytes regulate via Ca^{2+} signaling the blood flow in the brain [113]. Additionally, astrocytes respond very differently to the type of ECM proteins; as an example, regrowth of scratch wounds was halted in astrocytes cultured in fibronectin but not on laminin or tenascin C. Interestingly, IL-1b-challenged astrocytes behave very differently on fibronectin, laminin or tenascin C [114]. More specifically, challenged astrocytes increased proliferation when cultured in fibronectin while growth was arrested on laminin.

Geometry is also pivotal for the physiological state of astrocytes. 3D *in vitro* models showed that astrocytes are less reactive in 3D vs. conventional 2D [115]. Additionally, 3D ECM composition and collagen concentration were reported to influence the reactivity of astrocytes (in terms of GFAP expression). Interestingly, this study also reported that the number of astrocytic processes depends on collagen concentration [116]. More studies corroborate that astrocytes in 3D are less reactive than in 2D [115]. Astrocytes are highly permissive to ECM protein composition.

Chapter 4

Conclusions

Astrocytes are an integral part of the CNS. Historically, they have been misidentified as non-cellular components of the brain. What was once considered mere neurotransmitter cleaners, now astrocytes are recognized as a truly versatile brain cell type. Recent studies point out astrocyte's entanglement in a myriad of processes, both in brain homeostasis and neuroinflammation. This study presents the first inflammatory-responsive human iPSC-derived astrocytes. (Paper I and Paper II). The most rudimentary readouts for iPSC-derived astrocytes are cytokine secretion and phagocytosis. In this study, we further confirmed that hiAstrocytes secrete astrocyte-associated cytokines. More importantly, this study demonstrates that hiAstrocytes upregulate ICAM-1 upon inflammatory challenges. The importance of ICAM-1 is that it can facilitate direct communication with other immune cell types, such as monocytes and microglia.

Additionally, the importance of glutathione synthesis and secretion further expands on the astrocytic phenotype of hiAstrocytes. Astrocytes synthesize and secrete glutathione taken up by neurons, and paper I reports on the synthesis and secretion of glutathione from hiAstrocytes. The level of secretion and synthesis is on par with levels of glutathione that HFA synthesize and secrete. The similarity of glutathione secretion between hiAstrocytes and HFA adds to the *in vivo* relevance of hiAstrocytes

Paper III focused on how cell signaling can be altered based on culturing vessels. Paper III compared conventional culturing systems (e.g., traditional well plates) with microphysiological systems (e.g., chips) in somatic iPSC reprogramming and neural induction. Paper III suggests that the culturing environment does not impact iPSC reprogramming efficiency. Importantly, RNA-seq reveals no detectable transcriptomic differences between iPSC reprogrammed in well and chips conditions. The data of Paper III contradicts what was shown previously [117], more specifically, that a microfluidic setting increases the reprogramming efficiency (compared to conventional culture wells).

Nevertheless, Paper III capitalizes on MPSs and reports for the first time the effects of a confined culturing environment on neural induction and, consequently, the generation of neural stem cells. Initially, paper III shows that the confined environment boosts the generation of the neuroectoderm. Secondly, MPSs impart some form of standardization in the inherently variable process of neural stem cell generation as we see that neural stem cells generated and propagated in chips cluster closely, while NSCs generated in wells appeared more spread out.

Paper IV and V showcase how advanced *in vitro* models can be applied. Specifically, paper IV focuses on the barrier properties of the iPSC-derived barrier model and reports on the permeability of a drug delivery system, specifically a nanostructured lipid carrier. Paper V reports on a 3D vessel-like structure that is strategically positioned next to a glioma 3D model; this study could pave the way to study the vascularization of gliomas.

Chapter 5

Outlook

The work presented in this thesis can serve as a stepping stone for further evaluations. Specifically, ICAM-1 expression is important in disease modeling, specifically for multiple sclerosis [118–120]. Further studies could be done solely on the expression of this protein under inflammatory conditions. To exemplify, patient lines could be generated from MS patients and differentiated to hiAstrocytes; then, by comparing to the control lines, we could elucidate astrocytes' involvement in this neurodegenerative disease. Monocytes and microglia can directly link with astrocytic ICAM-1 via integrins such as CD11a. Further experiments could focus on whether hiAstrocytes can secrete the monocyte chemoattractant protein 1 (MCP1 or CCL2) since it is an important component of the cascade of reactions in multiple sclerosis [121].

Further studies on paper III would potentially involve the downstream differentiation of neural stem cells and investigate how perhaps culturing conditions alter neural stem cells' neurogenic/gliogenic potential. Even though an on-chip differentiation of these processes is tedious and perhaps unlikely, neural stem cells generated from chips and wells could show a varying propensity for neuronal or astrocytic differentiation. We could tie various pathways differentially activated in these two conditions with neuronal and astrocytic commitment with that knowledge. That would be fundamental in understanding neural stem cells' state since it is a heterogeneous population.

A follow-up study of paper IV could potentially focus on the inflammatory aspect of the hiPSC-barrier model. Additionally, another follow-up study could potentially focus on the mechanism that these drug delivery carriers use to pass through the barrier. Finally, the interesting platform generated in paper V can be used to evaluate more advanced studies. For example, CNS-relevant endothelium could be used instead of HUVECs. Additionally, astrocytes could be used instead of U87, along with amyloid-beta proteins, which are ubiquitous in Alzheimer's disease, to study the glymphatic system in detail and further characterize astrocytes' involvement in waste clearance. Furthermore, to further study the glymphatic system, sleep-inducing chemicals could be used since it is postulated that waste clearance is at its peak during sleep.

Acknowledgments

I would like to thank my supervisor, Anna Herland, for accepting me into her group, entrusting me with these projects, and being a beacon of inspiration throughout my Ph.D. studies. I would also like to thank Göran Stemme for creating a friendly and welcoming atmosphere at the Micro and Nanosystems division that had a positive impact during the tough times of the Ph.D. I would also like to thank the advance reviewer of this thesis, Håkan Jönsson, for his valuable feedback.

Next, I would like to extend my gratitude to Polyxeni (Xenia) Nikolakopoulou. Xenia, your enthusiasm for science and your positive disposition greatly influenced my psyche during the years I have known you. Enthusiasm and hope are contagious, so keep it up, spread it to the world, and always remember to take a moment to breathe. I will always be grateful for your mentorship, help with all my projects, and for being a great friend.

Isabelle, I would like to thank you for your friendship and company throughout these; you made Stockholm feel like a home away from home. I will never forget the endless days we spent in the little dungeon, as we called the cell lab at FPT. Your positive outlook on life is something that I aspire to adopt.

Emre, my office mate, thank you for your friendship and our interesting conversations. Serguei and Pierre merci pour votre amitié. All the people from the Herland lab, past and present members: Saumey, Sebastian, Julia, Thomas, Laura, Sara, Erica, Lily, Hanie, Julia and Rohollah; you have been all wonderful colleagues and I hope we cross paths again in the future.

I would also like to thank Rick, Dimitri, and Lucille. You were all amazing master students; you significantly contributed to my projects and helped me develop as a supervisor/ mentor.

Mattias, thank you for being there for me and providing support, and warm and happy memories. I also thank you for your linguistic and philosophical evaluation of my thesis.

Christiana, my sweet friend, thank you for being a good friend and a much-needed break for me, outside the craziness of writing papers and doing experiments.

To all my friends inside and outside of Sweden: Louiza, Jeanne, Eirini, Ioanna and Rena. Thank you all for your lifelong friendships.

Finally, I would like to thank my family. My mom, Marilena Gerontas and my sisters, Vaso, Eleni and Theodora. You have been such an inspiration to me, I would not have been where I am today without your support and belief in me. Σας αγαπώ. Dad, I hope you would have been proud of me.

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