Human iPSC-derived neuronal networks

Development and application for compound evaluation

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Cover illustration: *Wired*

The cover composite-images are illustrations of adherently growing human iPSC-derived 3D neural aggregate (front) and single cells (back), where beta-III-tubulin-positive neurons (blue) and DAPI-nuclei (red) are visualized. Schematic drawing on the front cover represents a microelectrode array. Imaged and processed by Julia Izsak with design help from Zsombor Sándor.

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"Discovery consists of seeing what everybody has seen and thinking what nobody has thought."

Szent-Györgyi Albert

ABSTRACT

Research on human brain development and function in health and disease has been hampered by limited access to primary human tissue and limited translatability of animal studies. This knowledge gap is encouraging the use of human induced pluripotent stem cell (hiPSC)-derived neural *in vitro* models. The current hope is that person-specific hiPSC-based *in vitro* models for human brain development and neuronal network function will increase the success in translating research results from bench to bedside. The aim of this thesis was to characterize and validate a person-specific human iPSC-based neural *in vitro* model to study the development, properties, and pharmacological modulation of human neuronal networks. In the first article we presented a procedure to generate 3D neural aggregates comprising astrocytes, oligodendrocytes and highly functional neurons that generated synchronous neuronal networks in less than three weeks. Further, by culturing hiPSC-derived 3D neural aggregates in human cerebrospinal fluid (hCSF), we demonstrated in article II that this adult brain-like milieu promotes morphological and functional maturation. Although hCSF is superior to currently used cell culture media, it has very limited availability for routine cell culturing purposes. This motivated the search for soluble factors that can mimic the observed maturational effects. In article III, we identified TGF-β1 as a physiologically relevant factor that can suppress proliferation and enhance neuronal and glial differentiation in a human 3D neural *in vitro* model. In article IV, we utilized this optimized model to provide insights in how therapeutically effective and overdose concentrations of lithium influence human single neuronal and network function. We showed that epileptiform discharges caused by overdose concentrations of lithium were suppressed by the antiepileptic drug Perampanel. The demonstrated functional impact of clinically relevant pharmacological compounds on human neuronal network function represents a proof-of-concept for the enhanced translational value of the human 3D neural aggregate *in vitro* model. The work presented in this thesis advances the field with a fast functional isogenic *in vitro* hiPSC-derived neuronal network model with improved physiological relevance and applicability for drug evaluation. Hopefully, our findings will bring the field of neuroscience closer to more translatable modeling and more successful clinical trials in the future.

Keywords: human induced pluripotent stem cell, neuronal network, microelectrode array, *in vitro*

POPULÄRVETENSKAPLIG SAMMANFATTNING

Forskningen kring den mänskliga hjärnutvecklingen och hjärnsjukdomar försvåras på grund av den bristande tillgången till viabel hjärnvävnad. En annan orsak till färre framsteg i fältet är att resultat från djurmodeller har varit svåra att överföra till den mänskliga hjärnan och klinisk praxis. Med hjälp av den Nobelprisbelönade teknologin för framtagning av pluripotenta stamceller har vi fått möjlighet att omvandla patientspecifika somatiska celler till pluripotenta stam celler. Pluripotenta stam celler ger möjlighet att framodla mänsklig hjärnvävnad från enskilda personer. I denna avhandling har vi utvecklat ett human-specifikt reduktionistiskt modell-system genom att odla fram patient-specifika neuronala kulturer. Dessa kulturer speglar stadierna av tidig hjärnutveckling och genererar spontan synkron nätverksaktivitet efter 2–3 veckor i odling. Även om denna metod använder biologiskt relevanta mänskliga celler, så odlas cellerna i kommersiella odlingsmedier som kritiseras för att inte vara fysiologiska. Vi har därför testat att odla cellerna under de mest fysiologiska förhållanden, dvs. i mänsklig cerebrospinalvätska. Vi har visat att cerebrospinalvätskan accelererar cellmognad samt funktionell utveckling i cellerna. Trots att cerebrospinalvätska visar sig vara förmer än de kommersiella odlingsmedier, är den inte tillgänglig för rutinmässig cellodling. Det är därför viktigt att hitta andra fysiologiskt relevanta faktorer som kan efterlikna effekten av cerebrospinalvätska på odlade nervceller. Genom att utvärdera andra fysiologiskt relevanta faktorer som skulle tillsättas till det kommersiella odlingsmediet, har vi optimerat odlingsförhållanden. Detta optimerade modellsystem öppnar upp för möjligheten att testa behandlingsmetoder på mänsklig hjärnvävnad *in vitro*. Vi använde modellen för att analysera effekten av klinisk relevanta läkemedel, som lithium och Perampanel och bekräftade att modellen är lämplig för prekliniska läkemedelsstudier. Resultaten i denna avhandling visar att hjärnceller framodlade från pluripotenta stam celler kan användas för att minska klyftan mellan preklinisk och klinisk vetenskap. Med detta hoppas vi att modellen kommer att kunna bidra till bättre framsteg i behandlingar inom neuropsykiatrin.

LIST OF ARTICLES

This thesis is based on the following peer-reviewed published articles, referred to in the text by their Roman numerals.

I. Izsak, J., Seth, H., Andersson, M., Vizlin-Hodzic, Dz., Theiss, S., Hanse, E., Ågren, H., Funa, K., Illes, S.

Robust Generation of Person-Specific, Synchronously Active Neuronal Networks Using Purely Isogenic Human iPSC-3D Neural Aggregate Cultures.

Front Neurosci. 2019; 13:35

II. Izsak, J., Seth, H., Theiss, S., Hanse, E., Illes, S.

Human Cerebrospinal Fluid Promotes Neuronal Circuit Maturation of Human Induced Pluripotent Stem Cell-Derived 3D Neural Aggregates.

Stem Cell Reports. 2020; 14(6):1044-1059

III. Izsak, J., Vizlin-Hodzic, Dz., Iljin, M., Strandberg, J., Jadasz, J., Olsson Bontell, T., Theiss, S., Hanse, E., Ågren, H., Funa, K., Illes, S.

TGF-β1 Suppresses Proliferation and Induces Differentiation in Human iPSC Neural *in vitro* Models.

Front Cell Dev Biol. 2020; 8:571332

IV. Izsak, J., Seth, H., Iljin, M, Theiss, S., Ågren, H., Funa, K., Aigner, L., Hanse, E., Illes, S.

Differential acute impact of therapeutically effective and overdose concentrations of lithium on human neuronal single cell and network function.

Transl Psychiatry. 2021; 11(1):281

CONTENT

ABBREVIATIONS

3D	Three-Dimensional
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ESC	Embryonic Stem Cell
FGF	Fibroblast Growth Factor
GABA	Gamma aminobutyric acid
iPSC	Induced Pluripotent Stem Cell
hiPSC	Human Induced Pluripotent Stem Cell
hCSF	Human Cerebrospinal Fluid
MEA	Microelectrode Array
PEDOT-CNT	poly-3,4-ethylene-dioxythiophene-carbon nanotube
RA	Retinoic Acid
SFEB	Serum-free Floating Embryoid Body
TGF	Transforming Growth Factor
TTX	Tetrodotoxin

1 INTRODUCTION

1.1 HUMAN PLURIPOTENT STEM CELLS: THEIR PAST AND HOPE

The high potential of pluripotent stem cells was probably first recognized in 1981, when researchers successfully isolated and cultured *in vitro* pluripotent stem cells from inner cell masses of mouse blastocysts (Evans and Kaufman, 1981; Martin, 1981). These cells were called embryonic stem (ES) cells. ES cells are characterized by three major properties: (i) they are derived from the preimplantation embryo (blastocyst), (ii) they are capable of prolonged undifferentiated self-renewal, and (iii) they have the potential to differentiate into all three germ layer-derived cell types. Having achieved a large increase in scientific knowledge on rodent ES cells, a further milestone in the field was the isolation and culturing of human ES cells in 1998 (Thomson et al., 1998). This technology opened completely new ways of studying early human development *in vitro* by using healthy human cells. However, this discovery also sparked a chain of concerns related to ethical issues. Most of these ethical concerns were circumvented through the breakthrough discovery of induced pluripotent stem cells in 2006. Shinja Yamanaka *et al.* discovered how to return adult mouse fibroblasts into pluripotent stem cells *in vitro* by using four defined transcription factors (OCT4, SOX2, KLF4 and c-MYC). The year after this pioneering mouse cell-based work, Yamanaka and in parallel Thomsons group described the successful reprogramming of human fibroblasts into human induced pluripotent stem cells (iPSCs) (Takahashi et al., 2007; Yu et al., 2007). This methodology enabled an unlimited supply of human pluripotent stem cells, from any living person with reduced ethical concerns.
In general, iPSCs and ES cells are known to have comparable characteristics: similar (i) morphology, (ii) global gene expression pattern, (iii) proliferation properties, and (iv) differentiation potential (Takahashi and Yamanaka, 2016; Wernig et al., 2007). These iPSC characteristics provide great hopes for the stem cell field: human pluripotent stem cells as *in vitro* tools to study the principles of human development, to reach the goal of person-specific disease modeling, to do pharmacological studies in a human-cell-based manner and to use *in vitro* grown patient-specific cells for transplantation and regenerative medicine. However, since the epigenetic signature of patient cells gets lost as a consequence of the reprogramming procedure and prolonged cultivation, the rationale to use iPSC for modeling age-related diseases, e.g. Alzheimer´s disease, has been questioned (Penney et al., 2020; Ravaioli et al., 2018; Roessler et al., 2014).

Nevertheless, human PSCs (ESCs and iPSCs) have the theoretical potential to be used to obtain any cell type of the human body *in vitro*. This differentiation potential combined with presumably unlimited proliferation capacity *in vitro* is particularly valuable for organ tissues with low regenerative potential, limited access, and no adequate alternative cell resources available, such as the human brain.

1.2 MODELING HUMAN NEURAL DEVELOPMENT *IN VITRO*

Ever since human PSCs became available, there was a high interest in differentiating them to neural cells to study human brain development and function *in vitro*. The protocols of *in vitro* neural induction and differentiation are based on the known *in vivo* patterning cues and processes revealed in animal-based research. For instance, the most well-studied morphogens that are expressed during *in vivo* cortical development and are used for *in vitro* neural differentiation purposes are retinoic acid (RA), bone morphogenic proteins, transforming growth factors (TGF), fibroblast growth factors (FGF), Wingless-Int and sonic hedgehog (Tiberi et al., 2012; Wilson and Houart, 2004). Sources of extracellular cues involved in *in vivo* corticogenesis are organizing centers in the developing brain, such as the floor plate, roof plate or cortical hem. Beyond this paracrine signaling, autocrine signaling by neural progenitors, neurons, and astrocytes contribute to brain development. Furthermore, morphogens can derive from the surrounding meningeal wall (e.g., RA), and can originate from the cerebrospinal fluid (e.g., FGF2, TGF-β) (Tiberi et al., 2012).

Intriguingly, *in vitro* differentiation of pluripotent stem cells towards neural stem cells and more mature neural progenies resembles aspects of *in vivo* cortical development (Gaspard and Vanderhaeghen, 2011; Mertens et al., 2016; Suzuki and Vanderhaeghen, 2015). The progressive stages of neural development are the following: (i) neural induction, i.e., neuroectodermal differentiation of pluripotent stem cells to neural stem cells, (ii) proliferation and regional specification, (iii) neuronal and glial commitment and (iv) terminal neuronal and glial differentiation and maturation, leading to (v) neuronal network formation.

The first *in vitro* neural induction of human ES cells showed that human pluripotent stem cells can be successfully differentiated to neural stem cells (Zhang et al., 2001). Neural stem cells are typically organized in so called neural rosette structures, the *in vitro* equivalent of the *in vivo* neural tube. Later studies using mouse and human ES cells demonstrated that these *in vitro* acquired neural stem cells have powerful intrinsic differentiation and

organizational properties (Eiraku et al., 2008; Gaspard et al., 2008). In particular, neural stem cells were shown to sequentially differentiate to layer-specific cortical progenitors of the developing cortex, recapitulating early steps of corticogenesis. In detail, these cultures contained nestin-positive ventricular zone-like cells, reelin-positive Cajal-Retzius cells, TBR1- and reelin-positive subplate-like cells, and cortical plate-like cells with sequentially born deep and upper layer cortical neurons as well. Since these *in vitro* models are meant to reflect properties of *in vivo* neuronal development, it is not surprising that besides their morphological and structural properties, their functional characteristics are similarly important.
Studies focusing on the functional properties of human ES cell-derived neurons showed that these *in vitro* differentiated human neurons exhibit characteristic Na^+ and K^+ currents and generate action potentials upon current injection (Erceg et al., 2008; Johnson et al., 2007). Furthermore, they have been shown to integrate functionally, i.e., establish synaptic connectivity when transplanted to rodent brain (Koch et al., 2009).

1.3 HUMAN NEURONAL CELL AND NETWORK FUNCTION *IN VITRO*

A collective aim in neuroscience is to understand the intriguing functional development and organization of neurons and neuronal networks. This collective drive led to several advances in technologies to assess and monitor aspects of functional neuronal development and communication.
Neuronal cell and network activity can be measured at different levels, i.e., (i) microscale level, assessing single cell and synapse level, (ii) mesoscale level, measuring neuronal population function and (iii) macroscale level, recording functional connectivity and communication between different brain regions. At microscale level, neurons can be assessed using the patch clamp technique (Sakmann and Neher, 1984). By intracellularly placed patch electrodes, it is possible to measure large voltage changes or currents during an action potential, but even subthreshold changes originating from ion channels and synaptic input (figure 1, left). However, the patch clamp technique is laborious and leads to a sequential loss of cell or tissue viability over the recording time. An alternative approach that offers unique information on this microscale neuronal activity, is measuring voltage changes in the vicinity of an electrophysiologically active neuron without breaching its cell membrane. These extracellular voltage changes during an action potential (also referred to as “spike”) can be measured by extracellular electrodes (microelectrodes) close to a neuron (Chiappalone et al., 2019). This allows non-invasive recording from neurons over long time. The use of extracellular microelectrodes began

with the application of single unit metal electrodes (Gesteland et al., 1959; Weale, 1951). The approach appeared promising and led to a fast advance in increasing the number of observed neurons by the introduction of more recording sites, a technique called microelectrode array (MEA, figure 1, right) (Thomas et al., 1972). Besides measuring single neurons, MEAs are also useful to measure mesoscale activity, giving information about electrophysiological properties of neuronal populations and inter-neuronal connectivity.

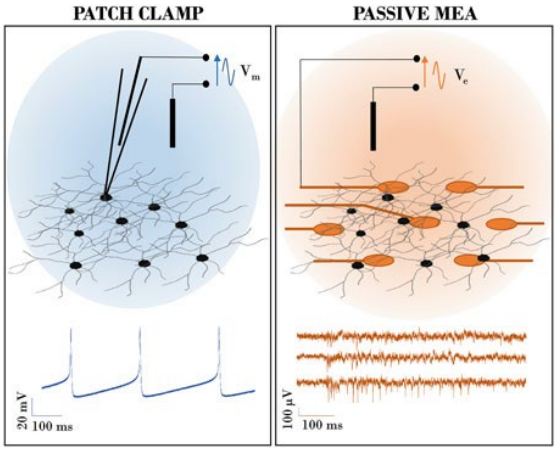

Figure 1 *In vitro* electrical recording methods
Schematic diagrams depicting current methods for *in vitro* electrical recordings: patch clamp on the left and MEAs on the right. Adapted from Chiappalone et al 2019.

The MEA field advanced rapidly leading to commercially available recording substrates with increasing numbers of recording electrodes in different types of cell culture chambers. This technology laid the foundation for functional examination of *in vitro* neuronal networks and opened opportunities for assessment of neuronal communication between spatially distant neurons. Planar MEAs became a popular tool to assess neuronal network activity *in vitro*, using diverse cell sources, starting from rodent primary cell preparations (Arnold et al., 2005; Chiappalone et al., 2006; Gopal and Gross, 1996), rodent slices (Egert et al., 2002; Karpiak and Plenz, 2002; Shimono et al., 2000), and human brain slices (Dossi et al., 2014; Hsiao et al., 2015; Wickham et al., 2020). A common hallmark of *in vitro* neuronal networks is a preserved self-organization capacity: they fire spontaneous action potentials, form synaptic connections, and develop synchronized neuronal network activity over time (Chiappalone et al., 2006; Luhmann et al., 2016; Wagenaar et al., 2005). When pluripotent stem cell-derived neurons became available, it was an interesting question whether this preserved property of neuronal network development could be reproduced in stem cell-derived cultures. The first studies describing successful development of spontaneous synchronized neuronal network activity from mouse embryonic stem cells were published in 2007 (Ban et al., 2007; Illes et al., 2007), followed by a study using human ES cell cultures in

2009 (Heikkilä et al., 2009). These studies proved that even *in vitro* differentiated stem cell-derived neurons are self-organizing in synchronized neuronal networks. In detail, mouse and human ES cell-derived neurons showed spontaneous single spiking activity after one week in culture, which developed sequentially into synchronous neuronal network activity within four weeks. Synchronous neuronal network activity is a collective electrophysiological behavior of synaptically interconnected neurons, with simultaneous spiking, detectable at several recording sites. Synchronous activity is also recognized as "network burst" or "population burst". ES cell-derived neuronal network bursts were shown to depend on neuronal excitability, glutamatergic and GABAergic synaptic communication (Heikkilä et al., 2009; Illes et al., 2007). Thus, ES cell-derived neural cultures reproduce major steps of neuronal network development, and they show pharmacological responses *in vitro.* These characteristics make them a suitable and valuable model system for studying the development and function of neuronal networks *in vitro*. The advent of human iPSC technology expanded the cell source of *in vitro* neurons with unlimited and person-specific cells paving the way to study the function of human neurons and neuronal networks, including pharmacological manipulation.

1.4 THE HOPE OF HUMAN IPSC-DERIVED NEURONAL NETWORKS ON A CHIP

Use of human iPSC-derived neural models has been proposed as a hope to improve pre-clinical modeling of neurological and psychiatric disorders as well as to advance drug discovery and development in the field (Ko and Gelb, 2014). Pharmacological testing and drug development in neurology and neuropsychiatry have been facing a low success rate. For instance, in comparison to other therapeutic areas, neurology and psychiatry show one of the lowest success rates in clinical trials (Hay et al., 2014; Howe et al., 2018). This low success rate is often attributed to species-specific differences and poor translatability of pre-clinical data acquired from animal models (Dawson et al., 2018; Hyman, 2014). Human brain slice preparations were developed to address this issue and enable studies in human-cell-specific models (Hsiao et al., 2015; Jones et al., 2016; Wickham et al., 2018). Moreover, resected human brain tissue has proven to be useful for evaluating antiepileptic treatments on human neuronal populations (Wickham et al., 2019). However, the epileptic nature of resected tissues limits their potential for broader use in studying physiological or other pathological human brain function properties and their response to pharmacological compounds. Another promising avenue for human-specific modeling is represented by human iPSC-based functional

neural assemblies (Engle et al., 2018; Ko and Gelb, 2014). Advantages of using human iPSC-based cultures for central nervous system modeling and drug evaluation are the following: (i) they are human cells, which can be patient-specific and disease-specific, (ii) they can be used to study brain developmental aspects, (iii) they have the potential to obtain multiple brain cell types, e.g. neurons, astrocytes and oligodendrocytes, from the same human tissue sample, (iv) they represent a nearly unlimited cell resource, as is required for upscaling. On the other hand, their potential disadvantages should also be mentioned, which are: (i) *in vitro* induced genetic mutations, (ii) unphysiological culturing environment, (iii) time-consuming and expensive culturing, (iv) relatively new technology with the need for standardization. Moreover, the robust generation of functional neuronal networks comprised of neurons and glial cells endogenously generated from the same human iPSC source had not been achieved and was a particular challenge when I started my PhD project.

Combining human iPSC-derived neurons with MEAs enables the functional study of neuronal network development from the appearance of single spikes to the development of correlated neuronal network activity. Further, person-specific neuronal networks (figure 2) can be potentially used for genetic manipulations and neuropharmacological compound testing.

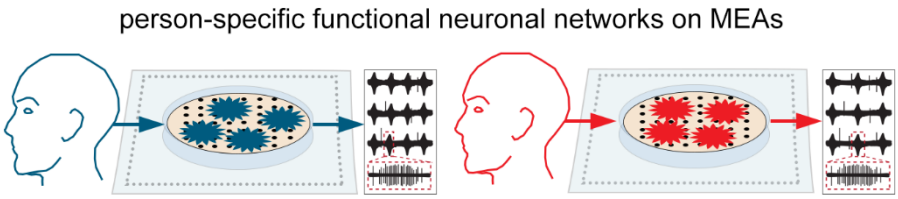

Figure 2 Person-specific neuronal networks on MEAs
Schematic drawing depicting the fusion of hiPSC-technology and MEAs, offering the possibility to study person-specific neuronal networks *in vitro*.

Nevertheless, reproducibly generating human induced pluripotent stem cell-based functional neuronal circuits, solely obtained from single individuals, poses challenges to achieve personalized and patient-specific functional neuronal *in vitro* models.

1.5 CHALLENGES

At the time when this PhD project started, human iPSC-derived neurons and neuronal networks required long time (3—8 months) to achieve highly synchronous networks (Amin et al., 2016; Odawara et al., 2016). In general, the long timeframe was attributed either to lack of certain components in culture media that would promote maturation (Dodla et al., 2010), or to the inherent nature of human brain development which is thought to take longer time compared to other primates (Lancaster et al., 2013; Mora-Bermúdez et al., 2016; Odawara et al., 2014). For instance, the appearance of astrocytes in stem cell-derived *in vitro* cultures was described to take 3—6 months, coinciding with the time required for neuronal and network maturation (Krencik et al., 2011; Paşca et al., 2015; Sloan and Barres, 2014). Interestingly, co-culturing human iPSC-derived neurons with rodent or human astrocytes facilitated the long-term culturing and maturation of neurons and networks (Fukushima et al., 2016; Johnson et al., 2007; Kuijlaars et al., 2016; Lam et al., 2017; Odawara et al., 2016; Shi et al., 2012). However, co-culturing with external rodent or human astrocytes does not support a true person-specific modeling and drug testing *in vitro* platform. This issue motivated the need for isogenic model systems where neurons and astrocytes are generated from the same patient cell source. Another issue, which made the system rather unphysiological, was the sub-optimal ion composition of cell culture media. To address this, Bardy *et al.* designed the BrainPhys media with adjusted composition and ion concentrations rather matching human cerebrospinal fluid (hCSF) (Bardy et al., 2015). Another concern in the field of iPSC-derived neural *in vitro* models was substantial proliferation and overgrowth, which were indicative for immaturity. Application of neurotrophic factors or small molecules is commonly used to inhibit proliferation and promote differentiation (Kemp et al., 2016; Kirkeby et al., 2012). However, the use of synthetic small molecules might represent a rather unphysiological approach. To this end, the functional and morphological assessment of human neurons cultured in the *in vivo*-like milieu of healthy adult hCSF represented a promising way towards the discovery of additional physiologically relevant factors that might be required to enhance maturation of *in vitro* neural models as well as to improve neuronal network function *in vitro.*

2 AIM

The aim of this PhD project was to characterize and validate a person-specific human iPSC-based neural *in vitro* model to be utilized for studying human neural development and neuronal circuit formation, as well as to assess pharmacological modulation of human neuronal networks *in vitro*.

The work was divided into four distinct parts, each with a specific aim:

I. To characterize neuronal network function in person-specific human iPSC-derived neural aggregate cultures.

II. To evaluate the neuronal network function and cellular properties of human iPSC-derived neural aggregates exposed to the most physiological culturing media and adult brain-like milieu: human cerebrospinal fluid.

III. To evaluate the effects of a selected physiologically relevant differentiation factor on neural proliferation, differentiation, and neuronal network formation in human iPSC-derived neural cultures.

IV. To evaluate the applicability of this model by assessing the impact of clinically relevant compounds on human neuronal network function.

3 METHODOLOGICAL CONSIDERATIONS

The experimental procedures used in the thesis are described in the attached articles. Here I discuss the reasons behind the choices and decisions that were taken along the project journey.

3.1 CULTURING HUMAN IPSC LINES

Cultivation of human iPSCs under feeder-free adherent conditions has been mainly performed in two different culturing systems: the colony type and non-colony type culturing. In the standard colony type culturing protocol, cells are growing in clumps (colonies, figure 3A), when using the mTesR culturing system. In contrast, in non-colony type culturing, human iPSCs are dissociated into single cells and are grown as a confluent monolayer culture (figure 3B), when using the Cellartis DEF-CS culturing system. Since the master iPSC stocks used in this study were originally cultured by using the DEF-CS system, this non-colony type method was used in the beginning of the study.
Working with iPSCs, one difficulty rises from their tendency to spontaneously differentiate to different cell types of the three germ layers. Since all subsequent applications of hiPSCs, including neural induction, depend on starting material quality, it is essential to keep stem cell cultures free of differentiated cells. A common approach is to distinguish stem cells from differentiated cells by microscopic visual inspection. Usually, undifferentiated iPSCs have the following morphological appearance: compact colonies with well-defined edges where cells show large nuclei and small nucleoli with small cytoplasmic area (figure 3A, i, ii). In contrast, differentiated iPSCs appear as colonies with irregular edges, cells with smaller nuclei and larger cytoplasm (figure 3A, iii).

One drawback of using the DEF-CS culturing system was that cell morphology was hardly visible by inspection in the microscope (figure 3B, ii), which led to more heterogenous iPSC-cultures also containing differentiated cells, and to lower efficiency of the following neural induction. This problem triggered the switch to the standard colony type culturing system (mTesR), which was used in the later part of this project. In this colony type culturing system, differentiated cells become clearly visible by microscopic visual inspection

(figure 3A, iii), and they can be directly removed under microscope assisted pipette cleaning (manual removal of differentiated cells).

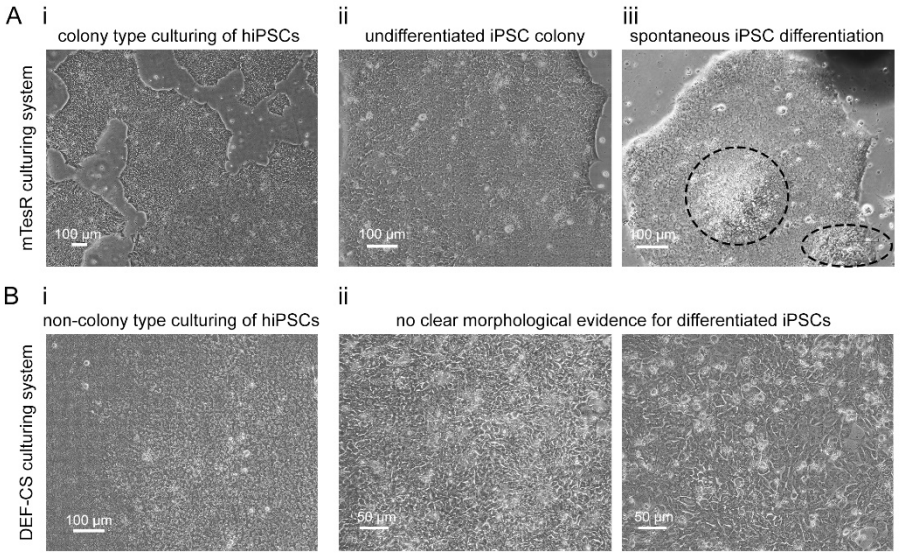

Figure 3 Morphological characteristics of human iPSCs in colony and non-colony type culturing systems
(A, i) Representative phase-contrast image of human iPSC-colonies cultured in mTeSR culturing system, showing (A, ii) undifferentiated and (A, iii) differentiated morphologies.
(B, i) Representative phase-contrast image of human iPSCs cultured in non-colony type culturing system, showing (B, ii) no clear morphological signatures of differentiated cells.

The ultimate goal of most human iPSC-derived model systems is their therapeutic use—either for cell transplants or drug evaluation studies. To fulfill this goal, iPSCs need to be cultured free of animal or other undefined sources. While mTesR growth medium is an animal-free, serum-free, and chemically defined medium (and so is the DEF-CS medium), Matrigel coating, which is one of the most widely used extracellular matrix for culturing human iPSCs, is a xenogenic substrate originating from Engelbreth-Holm-Swarm mouse sarcoma cells. To overcome this issue, in this study we used human recombinant laminin 521 as extracellular matrix, ensuring an entirely animal-free, serum-free, and chemically defined culturing environment. It has been shown and confirmed that 521 bio-laminin is suitable to support pluripotency of human iPSCs (Lu et al., 2014), as well as neural induction and differentiation (Hyvärinen et al., 2019; Lu et al., 2014; Niclis et al., 2017).

3.2 NEURAL INDUCTION

After achieving high-quality iPSC cultures, the next step was neural induction. A general high interest in this method led to a variety of neural induction protocols that were constantly improved and revised over time, creating a multitude of available neuronal differentiation protocols. The current gold standard of differentiating cortical neural stem cells from human iPSCs is the highly efficient dual SMAD inhibition protocol, described by Shi et al in 2012 (Shi et al., 2012). In this study, the combination of retinoic acid signaling with inhibition of the SMAD pathway leads to 95% cortical neural stem cells after 15 days in adherently growing human iPSCs. Another classical approach for neural induction is the so called embryoid body formation protocol, which implements the cultivation of iPSCs as free-floating aggregates. The dual SMAD inhibition protocol can be applied to both adherently growing or embryoid body type approaches. Both approaches lead to comparable outcomes in the cortical neural stem cell pool (Pauly et al., 2018).

In this thesis, both protocols were tested (figure 4), and the advantages and disadvantages of each are summarized in the following:

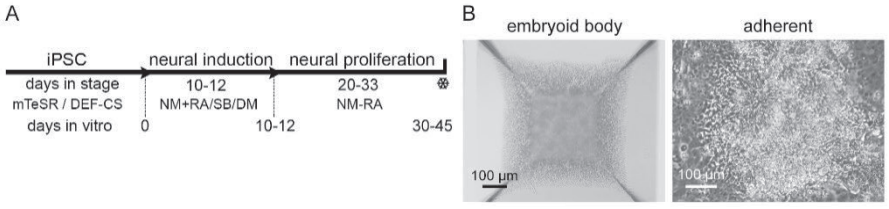

Figure 4 Neural induction
(A) Schematic drawing illustrating the used neural induction protocol. (B) Phase contrast images of neural induction done in embryoid body (left) and adherent (right) culturing type. NM, neural media; RA, retinoic acid; SB, SB 431542; DM, dorsomorphin.

In the adherent approach, where neural induction is started directly on adherently growing iPSCs, an important benefit is better visual control of cell growth and differentiation. Further, this approach implies lower costs since no special plates are needed. However, a pitfall of the method is potential cell detachment over time due to cell overgrowth. The embryoid body approach is usually done in so-called AggreWell plates to ensure similar aggregate sizes. One benefit of using AggreWell plates is scalability for larger throughput experiments. However, visual control of differentiation is hampered, and the costs of this approach are higher.
In this project, we opted for the adherent approach for cortical neural induction, partially due to a better visual control and partially due to its lower costs. The neural induction protocol is depicted in figure 4A.

3.3 NEURONAL DIFFERENTIATION IN 3D-NEURAL AGGREGATES

There is a plethora of literature highlighting the importance of a three-dimensional (3D) environment for *in vitro* cultured cells. It has been shown that cell-cell contact, and cell-extracellular matrix contact is essential for achieving more healthy and more *in vivo*-like cell cultures. For instance, Illes *et al.* showed in 2009 that neurons obtained from monolayer neural stem cell cultures displayed a limited differentiation capacity and functional maturation when compared to neurons obtained from more complex 3D serum-free floating embryoid body (SFEB) cultures (Illes et al., 2009). An emerging field that holds great translational promise is the human organoid field. Human organoids are human stem cell-derived self-organizing 3D culture systems, which mimic to a certain extent the architecture and physiology of human organs. Many organ-like structures have been grown in that way, including human brain organoids. For instance, a modified version of the SFEB culture approach has been used for the generation of the first brain organoids, described in 2013 by Lancaster *et al.* (Lancaster et al., 2013). Since then, they became a popular reductionist model system in neuroscience. Human brain organoids are a great model to study neurodevelopmental characteristics, such as microcephaly in Lancaster *et al.* 2013, however they show limited functionality up to five months in culture (Fair et al., 2020). This limited

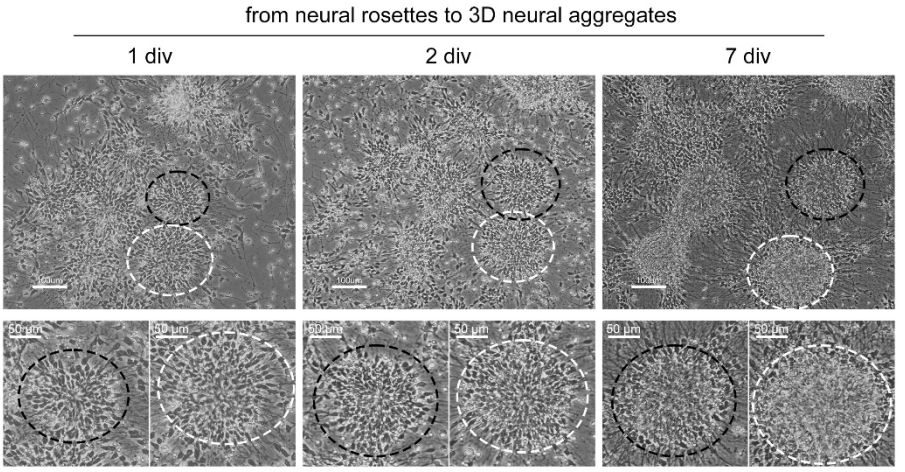

Figure 5 From neural rosettes to 3D neural aggregates
Phase contrast images showing the transition of neural rosettes towards 3D neural aggregates in time (from left to the right). The circles are highlighting individual neural rosettes and neural aggregates, shown at higher magnification below.
Scale bars: 100 µm above, 50 µm below.

functionality might be related to large sizes and deficient diffusion of nutrients and growth factors from the cell culture media (Qian et al., 2019).

In this project we implemented the 3D structure but used smaller sized (100—200 μm) 3D neural aggregates. These 3D neural aggregates are self-organized structures originating from neural rosettes (figure 5). For culturing 3D neural aggregates, we used BrainPhys media, to support the electrophysiological activity of neurons.

3.4 IMMUNOCYTOCHEMISTRY, CONFOCAL MICROSCOPY, AND IMAGE ANALYSIS

To characterize cell lines with respect to cell types and synaptic markers, immunocytochemistry (ICC) and confocal laser microscopy was used. Primary antibodies binding to neural stem cells (nestin, SOX2, PAX6, CD133), proliferating cells (Ki-67), postmitotic neurons (MAP2ab, bIII-tubulin), cortical pyramidal neurons (CTIP2, BRN2, SATB2, TBR1), interneurons (parvalbumin (PV)), synaptic markers (synapsin, vGlut1, PSD-95), glial cells (GFAP, S100β, AQP4, glutamine synthetase (GS), O4), and TGF-β receptors (TGF-βRI, TGF-βRII) were applied. Details about the antibodies and imaging are described in the articles.
Since most currently available antibodies for neural markers have been tested and used primarily in rodent tissue and cell culture specimens, staining human cells implies further challenges due to the lack of available data and references. Each antibody used in this project was tested by applying the antibody alone as well as with co-staining with an already confirmed antibody. For instance, for testing the anti-synapsin antibody (targeting the presynaptic membrane), we applied the following combinations: (i) anti-synapsin alone, (ii) anti-synapsin combined with anti-PSD-95 (targeting the postsynaptic membrane, thus confirming the localization of the protein) and (iii) anti-synapsin combined with bIII-tubulin (targeting neurons, thus confirming the neuronal specificity of the antibody), see supplementary figure 5 in **article II**. A further secondary-antibody control was used to exclude non-specific binding of secondary antibodies. For batch image analysis, ImageJ macro scripts were written to reduce analysis time. Image analysis results were manually reviewed to exclude false signal detection.

3.5 ELECTROPHYSIOLOGICAL CHARACTERIZATION

3.5.1 MICROELECTRODE ARRAYS

Planar MEAs are small chips with microelectrodes on a glass surface, allowing the culturing of bio-electrically active tissues and cells on the electrode surface. Thereby, electrical activity of cells (i.e., voltage change in extracellular environment) can be measured in a non-invasive way, with high temporal resolution (0.0004 s) over a longer time period.
Electrodes are generally made of electric conductors such as gold, titanium or recently PEDOT-CNT (poly-3,4-ethylene-dioxythiophene-carbon nanotubes). The general purpose of electrode design is low input impedance, generating the highest possible signal-to-noise ratio combined with excellent long-term biocompatibility. Each electrode can detect voltage changes from neurons in a radius of 50—100 µm, according to the manufacturer's specifications. Action potential amplitudes crucially depend on the distance between recorded neuron and nearest electrode; thus, this parameter should be used with caution. In this project, the 2100 MEA system from Multi Channel Systems was used. MEA plates were either 1-well MEAs (59 recording electrodes + 1 ground electrode per well) or 6-well MEAs (9 recording electrodes + 1 ground electrode per well). Electrodes were planar Ti/TiAu electrodes or PEDOT-CNT (with 30 µm diameter and 200 µm inter-electrode distance). This design ensures a good spatial resolution for the description of neuronal network activity.

3.5.2 HCSF EXPERIMENTS

For the hCSF experiments, frozen CSF samples were thawed and warmed to 37°C. In a pilot experiment, pH and osmolarity of hCSF samples were measured. Since pH showed basic values without gasing, every sample was supplemented with HEPES (10 mM end-concentration) for pH buffering.

3.5.3 DRUG EXPERIMENTS

For drug experiments, two types of experimental designs were used: the cumulative and non-cumulative drug application. Cumulative experiments were confirmed by non-cumulative experiments to exclude potential time-related effects on neuronal networks. For each experiment, a parallel vehicle control experiment was conducted. The used pharmacological compounds, concentrations, and their targets are summarized in the following table:

Table 1 Used pharmacological compounds

Compound	Concentration	Mode of Action
Tetrodotoxin (TTX)	500 nM	Voltage-gated sodium channel blocker
Picrotoxin	50 µM	GABA-A receptor blocker
Gabazine	50 µM	GABA-A receptor antagonist
GABA	5 µM	GABA receptor agonist
CNQX	50 µM	AMPA/kainate receptor antagonist
D-AP5	50 µM	NMDA receptor antagonist
Perampanel	1 nM–2 µM	AMPA receptor antagonist
LiCl	0.5 mM–10 mM	enhanced neuronal excitability, unclear mode of action

3.5.4 DATA ANALYSIS

For spike detection and population burst analysis, custom-made Delphi and MATLAB software were used, developed by our collaborator Stephan Theiss. To ensure proper detection of population bursts, each result set was compared to the raw recording traces. This semi-manual approach was confirmed to be more suitable for an appropriate and optimal population burst detection on MEAs (Mossink et al., 2021).

Since multi-unit extracellular measurement by MEA technology does not offer a detailed description of single cell or single synaptic activity, the technique was complemented with patch clamp recordings in the published articles. The patch clamp recordings were done by our co-workers.

3.6 ETHICAL CONSIDERATIONS

This PhD project includes the use of human samples. All experiments were carried out in accordance with guidelines and were approved by the local ethical committee at the University of Gothenburg. Informed consent was obtained from all subjects. The work with human iPSC lines was approved by "regional etikprövningsnämnden Göteborg, with DNR 172-08". As patients are not harmed, and they are not having any inconvenience by the one-time adipose tissue sample collection, there are no risks that can be mentioned regarding the patients involved in the project.
The procedure of hCSF sampling and sample application for research purposes was approved by "regional etikprövningsnämnden Göteborg, with DNR 942-12". CSF samples were collected via lumbar puncture from volunteers. The collection carries certain risks like discomfort and post-sampling headache. Subjects were informed about the risks and the sampling procedure was carried out by experienced specialists with the use of small atraumatic needles.

4 RESULTS

Human person-specific iPSC-derived neuronal networks are a very promising path towards a better understanding of healthy human neural development as well as neurodevelopmental disorders. Furthermore, such functional person-specific neuronal networks can serve as a basis for understanding effects and side effects of different neuroactive compounds on human cortex. This hope is big, but so are the challenges. The four articles summarized in this thesis represent the specific steps we went through to address hindering issues at the time when I started working on the projects included in this PhD thesis.

In 2016, when I first started working in the lab, one of the difficulties in the field of stem cell neuroscience was that human stem cell-derived neurons required long time (in excess of three months) to achieve synchronous neuronal networks *in vitro* (Amin et al., 2016) or required co-culture with external rodent or human astrocytes (Fukushima et al., 2016; Johnson et al., 2007; Kuijlaars et al., 2016; Lam et al., 2017; Odawara et al., 2016). However, co-culturing human iPSC-derived neurons with healthy human or xenogenic astrocytes bears the risk of masking a true disease phenotype, or diluting drug effects on hiPSC-derived neuronal networks, thus limiting their translational value. This motivated the first question of this PhD project:

Q1 | *Is it possible to achieve fast and reproducible differentiation of human iPSCs to synchronous neuronal networks by generating both neurons and astrocytes solely from one individual's cells?*

Previous work using mouse ES cells (Illes et al., 2009) and human iPSC-based proof-of-concept experiments conducted by Sebastian Illes demonstrated that hiPSC-derived neurons within a three-dimensional (3D) neural aggregate environment are capable of generating synchronously active neuronal networks on MEAs within a shorter time period (less than 3 weeks after plating, 55-65 days post iPSC-stage) (Illes et al., 2016). In **article I**, I evaluated the efficacy of this procedure by using three different hiPSC lines from healthy donors. I showed that within three weeks after seeding human 3D neural aggregates on MEAs, all three cell lines exhibited robust synchronous activity. The temporal development of functional neuronal network properties showed a well-defined and predictable pattern: from asynchronous (i.e., stochastic) spiking between 3—9 days, to partially synchronous between 7—22 days and exclusively synchronous (i.e., organized population bursting) network activity after day 22. Complementary immunocytochemistry and confocal imaging revealed that neurons within 3D neural aggregates show numerous PSD-95^{+}

synapses and are surrounded by endogenously developed human astrocytes. Further, I confirmed that synchronous network activity on MEAs depended on neuronal excitability as well as synaptic neurotransmission via excitatory and inhibitory synapses. Complementary patch clamp recordings conducted by our group members demonstrated that neurons were excitable, showed spontaneous action potentials and bursts, as well as spontaneous glutamatergic and GABAergic synaptic activity. To summarize, our approach allowed the combination of MEAs with person-specific human iPSC-derived 3D-neural aggregates to reproducibly study *in vitro* human neuronal network development and pharmacological response in a short time frame (within 3 weeks).

Still, an issue that we and others noticed was ongoing continuous proliferation of human iPSC-derived 3D neural assemblies leading to overgrowth and detachment from the culturing surface. These signs were indicative for immaturity of human neural models and their inability to achieve terminal differentiation. It was then an interesting question whether this immaturity was related to lack of media components that would promote maturation. To this end, the next question of the project was:

Q2 | *Can human iPSC-derived neural cultures achieve cellular and functional maturation in vitro when exposed to a physiologically relevant adult brain-like milieu?*

In **article II**, we described the function of hiPSC-derived neural aggregates exposed to the most physiological culture media: human cerebrospinal fluid. We demonstrated that hCSF triggered neuronal network maturation of human iPSC-derived 3D neural aggregates on MEAs within three days. Improved neuronal network activity was maintained over time and persisted even after removal of hCSF. This long-lasting functional effect indicated underlying hCSF-mediated long-term cellular and sub-cellular effects. Thus, we evaluated synapse and single-cell electrophysiological properties as well as cellular content and subcellular changes in neurons and astrocytes. By applying patch clamp recordings, we found that three days hCSF application significantly increased the number of synaptic inputs of neurons (both excitatory and inhibitory) and decreased their input resistance indicative of neuronal maturation. To obtain insights in the morphological properties of 3D neural aggregate cultures exposed to BrainPhys or hCSF, we applied immunofluorescent staining and confocal microscopy. We revealed that hCSF rapidly (within three days) triggered gliogenesis, synapse formation, neurite outgrowth, as well as suppressed proliferation of residing neural stem cells. Thus, human CSF induced a chain of cellular maturational processes that were

responsible for enhanced functionality on single neuronal and neuronal network level. Furthermore, by culturing 3D neural aggregates in hCSF for 28 days on MEAs, we revealed that all neuronal network parameters reached a stable plateau phase within 11 days. To summarize, these data demonstrate that human iPSC-derived neural cultures can adopt more mature properties, if they are exposed to an appropriate environment. Nevertheless, human CSF is not available for routine use in cell culture, thus other approaches are needed to achieve more appropriate and physiologically relevant culture conditions *in vitro.* The third question of this PhD project was:

Q3 | *Can we identify and evaluate physiologically relevant factors that are mediating neural stem cell differentiation and are suppressing proliferation?*

Application of neurotrophic factors or small molecules is commonly used to inhibit proliferation and promote differentiation in human iPSC-derived neural cultures (Kemp et al., 2016; Kirkeby et al., 2012). However, the commonly used synthetic small molecules, such as DAPT or PD0332991, are not physiologically relevant. A path to circumvent this issue could be the application of physiologically relevant factors that inhibit proliferation and promote differentiation of human neural stem cells *in vitro*. As part of the third article, we used human brain transcriptome database queries (Kang et al., 2011) to identify signaling proteins that are up-regulated during the early phases of cortical development and become down-regulated with neuronal and glial differentiation. We surmised that those signaling proteins might mediate the transition of neural stem cells into neurons or astrocytes during human brain development. One such candidate factor was TGF-β1. Based on the well described effects of TGF-β-signaling in neural stem cell proliferation and differentiation (Stipursky et al., 2014; Wachs et al., 2006), we opted to evaluate TGF-β1 as a prospective factor for regulating neural stem cell proliferation and differentiation in our *in vitro* human iPSC-derived 3D neural aggregate model. As a first step, we confirmed the presence of TGF-β receptors in human iPSC-derived neural stem cells and neural aggregate cultures by q-PCR, performed by a member of our group and by immunocytochemical investigation. Next, I used immunocytochemistry and confocal laser microscopy to assess whether the additional application of TGF-β1 to cell culture media would inhibit proliferation and promote differentiation in our model system. I revealed that additionally applied TGF-β1 was suitable to suppress proliferation and promote neuronal and glial differentiation in human iPSC-derived neural stem cell and 3D neural aggregate cultures. After morphological assessment, we were interested in whether this factor also influenced single neuronal and neuronal network functionality. A co-worker performed patch clamp recordings and I performed MEA assessment of iPSC-derived neural cultures

in the absence or presence of TGF-β1 additionally applied to the cell culture medium. We found no major differences related to TGF-β1 exposure neither on single cell, nor on neuronal network level. Thus, we deduced that TGF-β1 does not alter electrophysiological function of human iPSC-derived neurons and their networks. Since TGF-β1 is specifically up-regulated during early phases of human fetal brain development and its expression profile is reciprocal to synaptic markers and neurotrophic factors, it is reasonable to conclude that TGF-β1 is only involved in early neural developmental processes, i.e., regulation of proliferation and transition of neural stem cells into neurons. Later neural developmental processes are not mediated by TGF-β1 and require the presence of neurotrophic factors to promote functional maturation and synaptogenesis.

With this optimized model in place, the next question we asked was:

Q4 | *Can we use this optimized in vitro human model to assess the effects of clinically relevant pharmacological compounds on human neuronal network and single cell functionality?*

One of the major hopes related to human iPSC-derived neuronal networks is their use as drug evaluation platform in a more human-specific context. In **article IV** we aimed to address this question and investigated the effect of clinically relevant compounds on human single cell and neuronal network function.

Lithium (Li) salts have been successfully used in clinical practice, without exactly knowing what their mode of action is. Interestingly, Li has a narrow therapeutic index, meaning that if the plasma concentration is exceeding 1.2 mM, patients are more prone to develop side effects—including epileptic seizures (Ghadirian and Lehmann, 1980). Thus, to mimic this clinically relevant therapeutic window, we tested different Li concentrations and their effects on human neuronal network activity. We revealed that Li has indeed a concentration-dependent effect in our *in vitro* functional human neuronal model: therapeutically effective concentrations of Li (≤1 mM) increased overall network activity on MEAs, and it increased neuronal excitability on single cell level as shown by patch clamp experiments. In contrast, high concentrations (i.e., exceeding the therapeutic window; ≥ 2 mM) of Li changed the network firing behavior to activity patterns reminiscent of epileptiform network activity ("population super bursting"). We confirmed this observation by applying GABA-A receptor inhibitors to cultured hiPSC-networks: application of picrotoxin or gabazine elicited similar neuronal network firing patterns. Further, overdose concentrations of Li induced increased excitability,

and interestingly only in the presence of high Li concentrations, increased AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) current frequency could be observed. Since this overdose pattern could be related to a chemically induced, or "iatrogenic" epileptiform activity, with a change in AMPA-receptor mediated transmission, the next step was to evaluate a clinically relevant anti-epileptic drug (Perampanel) which acts via an AMPA receptor pathway. We showed that Perampanel, an AMPA receptor antagonist, successfully rescued the Li-induced epileptiform phenotype in a dose-dependent manner. To summarize, in **article IV** we provided insights in how therapeutically effective as well as overdose concentrations of lithium directly influence human neuronal function at synaptic, single neuron, and neuronal network levels. Furthermore, we showed that Perampanel suppresses pathological neuronal discharges caused by overdose concentrations of lithium in human neurons. With that we presented proof-of-concept data for the potential translational value of our humanized model system for drug evaluation.

5 DISCUSSION

In the last two decades, the research community witnessed a very fast expansion of cell culture techniques for culturing and differentiating human iPS cells and technologies to assess their structure and function. Still and all, this growing young field will see much more progress and advancement. The work summarized in this thesis moved the field forward by establishing a fast and reproducible human cell-based tool to study functional human neuronal network development, as well as its application for drug evaluation. We provided proof-of-concept data highlighting that human iPSC-derived neuronal networks represent a promising tool (i) to describe person-specific neuronal network development, (ii) to decipher morphological and functional effects of externally applied bio-samples (human CSF) on human iPSC-derived neuronal networks, (iii) to evaluate physiologically relevant factors influencing neural stem cell proliferation and differentiation, and (iv) to evaluate effects of clinically relevant pharmacological compounds in a purely isogenic *in vitro* human neuronal network model.
One key improvement that made this possible was the use of a 3D neural aggregate model. Although the field of stem cell-based *in vitro* modeling shows a trend to move towards 3D culturing, 2D cultures are still used and useful. Which criteria should be considered to select an appropriate model?

5.1 DIMENSIONS: 2D OR 3D

Choosing between 2D and 3D modeling depends largely on the research question. An interesting recent study explored the molecular and functional consequences of 22q11.2 deletion syndrome, a common genetic risk factor for neuropsychiatric disorders, such as autism spectrum disorder and schizophrenia (Khan et al., 2020). This study used iPSC-derived *in vitro* neural cultures, applying both 3D and 2D approaches. A clear functional phenotype was described related to changes in resting membrane potential, excitability, with disturbances on voltage-gated calcium channels. Interestingly, the phenotype was present in both 3D cultured and 2D cultured neurons. This study elegantly confirmed that in certain cases, simpler 2D cultures can be enough to elucidate such functional phenotypes. Thus, research questions addressing phenotypic aspects related to consequences of genetic mutations on neuronal development and function may possibly be answered by using 2D cultures. A similar conclusion applies for neuronal cultures derived from direct reprogramming, which are often cultured as 2D monolayers. These 2D

cultured cells have been also proven useful for mapping *in vitro* phenotypic changes in diseases with known genetic mutations (Mossink et al., 2021).
Other questions that require more complex organization and architecture would benefit from using 3D assemblies. For instance, aspects such as cell migration have been studied in more complex fused spheroids or brain organoids (Bagley et al., 2017; Birey et al., 2017). Further, some functional aspects such as emergence of oscillatory activity are believed to require 3D structure (Buzsáki et al., 2012). Studies using cortical organoids (Sharf et al., 2021; Trujillo et al., 2019) and cortical-ganglionic eminence fusion organoids (Samarasinghe et al., 2021) showed that 3D architecture enables the generation of complex oscillatory patterns.
Taken together, both systems (2D and 3D) are proven to be useful, and the "fit for purpose" approach is reasonable when choosing between the two. In this study, we used a "2.5D" middle ground approach, where the 3D clusters are smaller, and grow on a 2D surface. We showed in **article I** that this approach enables fast and reproducible development of synchronous neuronal networks. Further, we showed in **article II**, **III** and **IV** that this model gives the opportunity to study effects of extracellular cues, and pharmacological compounds in a more complex but still adherently grown model. Additionally, our model enables the study of complex activity patterns, such as network oscillations (data not shown, manuscript in preparation).

5.2 CELL SOURCE

The next important aspect that 3D culturing enabled was the endogenous generation of both neuronal and glial cells from the same human iPSC source. This seemingly simple improvement can have relatively major impact on our way to develop better models (or models at all) for human-specific neurological or neuropsychiatric disorders. The role of astrocytes in disease pathogenesis has gained more attention in recent years and has been shown to be a major player in neuropsychiatric disorders (Siracusa et al., 2019; Zhang et al., 2021). For example, a recent study demonstrated that astrocytes derived from bipolar disorder patient iPSCs were less supportive for neuronal function. Namely, neurons co-cultured with bipolar disorder astrocytes showed marked decrease in neuronal function on MEAs compared to control astrocytes (Vadodaria et al., 2021). Thus, previous approaches to culture human iPSC-derived neurons together with rodent or human astrocytes, might be inappropriate for the future (Fukushima et al., 2016; Johnson et al., 2007; Kuijlaars et al., 2016; Lam et al., 2017; Odawara et al., 2016). Besides a drawback of phenotypic masking, co-culture with external astrocytes

introduces an additional variability, complicating the interpretation of results even further (Mossink et al., 2021).
In **article I**, **II**, and **III** we demonstrated that *in vitro* neural cultures, grown in a more complex 3D environment develop astrocytes endogenously. Fast endogenous development of astrocytes in turn contributed to a fast functional development towards synchronous neuronal network activity within less than three weeks.

5.3 TIMELINE AND CULTURE ENVIRONMENT

It is a general notion that human neurons require longer time to mature both *in vivo* and *in vitro.* This phenomenon is often explained by a presumed differential internal clock of human neurons leading to delayed maturational processes (Kelley and Pașca, 2022; Libé-Philippot and Vanderhaeghen, 2021). For instance, the species-specific longer morphological and functional maturational timelines in human brain organoids or cortical spheroids (in excess of five months) are explained by cell-intrinsic genetic properties of human neurons (Fair et al., 2020; Paşca et al., 2015). Further studies conducted in experimental paradigms where human neurons were transplanted into mouse brains also showed major species-specific timeline differences. Xeno-transplanted human neurons took several months (up to 11 months) to mature in the mouse brain (Linaro et al., 2019).
Nevertheless, both *in vitro* brain organoid and xeno-transplanted human stem cell-derived neurons bear a particular risk: human cells are not exposed to an appropriate physiological environment. A recent study exploring gene expression profiles in brain organoids provided evidence for upregulation of cellular stress genes (Bhaduri et al., 2020). Interestingly, the observed cellular stress was alleviated when the cells were transplanted to mouse brain, confirming the role of environment in eliciting stress response. Still, even with a lower cellular stress, human neurons differentiated slower in the mouse brain than their transplanted mouse counterparts (Falkner et al., 2016; Michelsen et al., 2015). One can still ask, is the mouse brain environment physiological enough to support faster maturation of human neurons? An interesting, yet ethically questionable and technically challenging experiment would be to transplant human neuronal progenitors to the human brain and follow their electrophysiological maturation and developmental timeline. Since this experiment is virtually impossible to do in healthy adults, one approach to mimic the healthy adult *in vivo* brain environment is the application of human CSF. We have shown that by exposing the *in vitro* derived neural cells to the soluble factors that are surrounding a human neuron *in vivo*, the iPSC-derived neural cultures are indeed capable of faster maturation. In **article II,** we

demonstrated that upon a mature external stimulus, several morphological and functional maturational changes can occur only within three days *in vitro*. This suggests that the internal genetic clock can be manipulated by external cues and the developmental timeline can be compressed *in vitro*. Whether the observed hCSF-induced maturation in human iPSC-derived neural aggregates was also accompanied with reduced cellular stress would be an interesting question to address in the future.

Nevertheless, our results suggest that the maturation of human neurons and neuronal networks *in vitro* does not take several months and occurs rather fast, on a timescale of a few weeks as we showed in **article I** and **II**. In line with our results, a study comparing the development of human PSC-derived neuronal networks with primary rat embryonic cortical neurons, demonstrates a comparable timeline of the development of synchronous neuronal networks on MEAs (Hyvärinen et al., 2019).

Another argument against the hypothesis of a cell-intrinsic driven differentiation timeline of human neurons is the simple existence of subplate cells. Subplate cells are the first postmitotic neurons that are populating the *in vivo* embryonic cortex and are characterized by a fast maturation towards more mature-like electrophysiological properties (Molnár et al., 2020). Why would we assume that subplate cells have a faster cell-intrinsic differentiation and maturation program by default? I would rather imagine that the local niche within the subplate is different than the local niche in the cortical plate, leading to differences in gene expression and developmental timelines. It would be an interesting experiment to decipher whether cortical plate progenitors transplanted to subplate would mature faster, and if so to find out what this local niche is comprised of.

Overall, it is questionable whether the timeline differences in corticogenesis reflect true species-specific differences, or if they just simply reflect that mouse and human progenitors have different environmental requirements for their differentiation and maturation, leading to different *in vivo* developmental timelines. Of note, even though this timeline does not seem to be human-specific, still human brain cells show unquestionable differences to their rodent counterparts. For instance, human neurons and astrocytes have been shown to have larger cell sizes, more processes and larger dendritic arborizations leading to differences in activity and information processing ("computing") capacity (Mohan et al., 2015; Oberheim et al., 2009).

5.4 ACTIVITY-DEPENDENT DEVELOPMENT

Besides environmental cues and soluble factors that are shaping the development and maturation of neurons, further essential aspects to be mentioned are activity-dependent mechanisms. It has been shown that electrical activity of neurons can influence morphological developmental aspects such as cell differentiation, migration, apoptosis, synaptogenesis, and network formation (Kilb et al., 2011). In general, neuronal and neuronal network development is the result of a loop where the structure shapes the function, and the function in turn reshapes the structure (Luhmann et al., 2016). To this end, the maturational changes described in **article II**, that were attributed to potential soluble factors present in the hCSF, should be also discussed in relation to activity-dependent aspects. We and others have seen and shown that acute application of hCSF has an immediate effect on the activity of neurons *in vitro*: an acute increase in the spontaneous firing of neurons in comparison to artificial CSF (Bjorefeldt et al., 2015; Wickham et al., 2020). This acute activity change can potentially influence gene-expression and can contribute in turn to the observed morphological and functional maturation. For instance, neuronal activity has been shown to regulate glial differentiation (Cohen and Fields, 2008). Blocking spontaneous activity for 5 days by TTX in primary hippocampal cultures decreased the percentage of astrocytes and reduced GFAP expression. Neuronal activity is also known to have a role in dendritic development. Blocking synaptic transmission in hippocampus during the first postnatal week *in vivo* resulted in threefold reduction of dendritic branching (Groc et al., 2002). Thus, the effect of hCSF on hiPSC-derived neural aggregates is most probably related to both soluble factor-derived mechanisms and activity-dependent changes. To what extent one or the other is contributing to the observed effects is an interesting but challenging question to address. One way to potentially answer part of the question would be to apply hCSF in the presence of TTX and assess the morphological maturational changes related solely to soluble factors. Interestingly, in **article III**, the sole application of TGF-β1 elicited morphological changes, such as lower proliferation, and increased number of neurons and glial cells. However, these morphological changes by themselves were not enough to induce functional maturation—no significant electrophysiological changes could be observed after applying TGF-β1. This discrepancy might be explained by the lack of activity-related gene expression changes with sole application of TGF-β1. An interesting future experiment would be to apply an activity increasing condition, by electrical or chemical stimulation together with TGF-β1, to potentially mimic activity-dependent maturational aspects. In conclusion, elucidating the impact of activity-

dependent mechanisms contributing to maturation of human iPSC-derived neural cultures will be of a great interest in the future.

5.5 MATURITY

Since human iPSC-derived *in vitro* neuronal models are meant to reflect properties of *in vivo* neuronal development and function, it is not surprising that a common question in the field is how mature these models are. Transcriptome studies addressing human iPSC-derived neural cultures show a high cellular diversity, with varying cellular maturity, comparable to transcriptome profiles of early human fetal and mouse postnatal stages (Burke et al., 2020; Stein et al., 2014; Tanaka et al., 2020). This cellular diversity is present in our 3D neural aggregate model as well: we described that 3D neural aggregates are comprised of cell types starting from nestin and SOX2 positive neural stem cells to astrocytes, postmitotic neurons, as well as synapses. Interestingly, we demonstrated in **article II** that after 3 days exposure to hCSF, cultures still contained immature cell types such as neural stem cells, but also exhibited other cellular maturational aspects, such as increased neuronal outgrowth, higher number of astrocytes, neurons, and synapses. Further, hCSF induced the translocation of vGlut proteins from the cytoplasm to synapses (see figure 6E, III in **article II**), a process associated with neuronal maturation (Illes et al., 2009; Real et al., 2006). Nevertheless, it would be interesting to address in future studies, whether longer exposure of hiPSC-derived neural cultures to hCSF (1—2 weeks) would further enhance morphological maturational processes, such as significant reduction of neural stem cells.

A study exploring the morphological and functional maturity of human pluripotent stem cell-derived neurons showed that morphological neurodevelopmental phenotypes, such as soma size and complexity of neuronal arborization correlate with electrophysiological states assessed by patch clamp, targeting single cell functionality (Bardy et al., 2016). This study by Bardy *et al.* explored electrophysiological phenotypes of stem cell-derived neurons and defined five distinct neuronal maturational stages, based on action potential firing properties. These five action potential types were classified as immature (Types 1—3), transitional (Type 4) and highly functional (Type 5) neurons (see figure 1 in (Bardy et al., 2016)). Immature neurons showed abortive action potentials upon depolarizing current injection (see Type 1, figure 1d), which developed into single action potentials (see Type 2, figure 1d) followed by stages showing the ability to fire increasing numbers of repetitive action potentials (Type 3—5). Type 5 neurons were defined as a highly functional state of maturity. Interestingly, our results in **article III** showed that neurons cultured without or with TGF-β1, in absence of other

neurotrophic factors could only reach a single cell maturational level comparable to more immature Type 3 action potentials (compare figure 5B, v in **article III** with figure 1d in (Bardy et al., 2016)). When cultured with neurotrophic factors and hCSF, the action potential properties of our neurons were comparable to the highly mature neurons of Type 5 (see figure 7C in **article I**, and figure 5F in **article II**). Of note, although Type 5 is the latest detectable stage with respect to action potential morphology, other parameters can reflect further maturational steps. For instance in **article II**, although the morphology of evoked action potentials was indistinguishable between BrainPhys and hCSF (figure 5F), we found that the input resistance of neurons exposed to hCSF was significantly decreased, a feature commonly observed during neuronal maturation (Bardy et al., 2016; Ehrlich et al., 2012; Mongiat et al., 2009). Further, in parallel with action potential maturation, an increase in synaptic activity was also observed previously (Bardy et al., 2016), which is in line with our results in **article II**.

Synaptic activity is associated with functional maturation of neuronal networks. While the functional maturity of human iPSC-derived neurons is somewhat characterized on single cell level, the definition of functional maturity on the mesoscale neuronal network level is still vague and not well defined (Pelkonen et al., 2022). Although synchronized neuronal network activity is often considered as a sign of neuronal maturation and synaptic interconnectivity, it is not necessarily the endpoint of neuronal circuit development. Spontaneous synchronous activity is a basic developmental property of early cortical networks (Molnár et al., 2020) and different types of synchronous neuronal network activity patterns emerge during distinct brain developmental stages (Luhmann et al., 2016). With maturation, this early synchronized activity develops into oscillating neuronal networks *in vivo*, e.g., spindle burst or delta brush activity, which sequentially transforms into desynchronized activity as sensory input starts to emerge (Molnár et al., 2020). Since *in vitro* neuronal networks are lacking sensory stimuli, *in vitro* synchrony is often considered as stable state, being the result of the exploring dynamics of a system deprived of external input (Chiappalone et al., 2006; Wagenaar et al., 2005). Long-term culturing studies of human iPSC-derived neuronal networks showed that reaching an end-point with steady-state neuronal network activity requires over 300 days (Shimba et al., 2019) or 230 days when co-cultured with astrocytes (Odawara et al., 2016). Intriguingly, our results suggest that hCSF exposure brings the synchronous neuronal network activity to a plateau phase within 11 days (figure 3 in **article II**).

A further typical functional maturational feature of *in vitro* and *in vivo* neurons is the postnatal switch from excitatory to inhibitory GABA-A receptor mediated effect (Ben-Ari, 2002; Peerboom and Wierenga, 2021). Interestingly, our cultures show mostly inhibitory effect of GABA-A receptors: application

of GABA-A receptor antagonists in **article I** and **IV** resulted in the increase of population burst activity, suggesting that a larger proportion of GABA-A receptors are coupled to postnatal-like neurons, where GABA-A receptor mediated effect is inhibitory.
To summarize, although the "maturity" of single neuronal and network properties of human iPSC-derived neuronal networks are not clearly defined in the literature, they recapitulate major developmental characteristics which makes them a valuable model. Future efforts in describing and defining characteristics of "maturity" are needed.

5.6 REPRODUCIBILITY AND ROBUSTNESS

A major critique of iPSC-derived neuronal cultures is their arguable reproducibility and robustness (Engle et al., 2018). We showed in **article I** that three independent human iPSC-lines developed comparable functional hallmarks over time. In addition, I confirmed the described functional neuronal network formation and timeline by using additional hiPSC lines obtained from other healthy individuals as well as hiPSC lines obtained from bipolar disorder patients (unpublished). In general, control and disease-related functional phenotypes from *in vitro* differentiated neuronal networks on MEAs have been recently concluded as being reproducible (Mossink et al., 2021). Although we showed robustness and reproducibility of the differentiation, in **article I** and **IV** we noticed that there are certain parameters that show increased variability, and thereby, are less useful for describing differences between cell lines or drug applications. One such parameter is the "total spikes/min". In line with our observation, the study by Mossink *et al.* showed similar results (Mossink et al., 2021). Namely, while in **article I** we described a coefficient of variation of 51% for the total spikes/min, Mossink *et al.* described a similar coefficient of variation of over 50%. Even though the mean firing rate does not seem to be an appropriate parameter, the existing variability in the recorded signal can still be compensated by using more suitable parameters, such as network burst rate. A future study assessing the functional variability between different labs and protocols would be of a great interest. Additionally, standardizations in protocols and lab practices will be essential for improving future interpretations and translatability.

5.7 TRANSLATABILITY IN DRUG EVALUATION

The current collective hope is that using human patient-specific and disease-specific cell types will lead to improved clinical predictability of pharmacological compounds (Engle et al., 2018). Human iPSC-derived neurons and neuronal networks are good candidates to improve clinical predictability. Nevertheless, proof-of-concept and validation studies are still required. Our proof-of-concept **article IV** shows that indeed, these iPSC-derived functional neuronal networks can reproduce aspects of therapeutical and toxic effects of clinically relevant drugs. The next piece of the puzzle to be solved is their scalability and reproducibility on larger scales. While 2D cultures have been shown to be scalable for pharmacological screens (Wang et al., 2017), and a recent 3D spheroid and calcium dye-based culture system showed to be applicable for screening smaller libraries (Woodruff et al., 2020), establishment and validation work are still needed.

6 CONCLUDING REMARKS

The current thesis has added new knowledge to the growing field of iPSC-based neural modeling. First, we have shown that human iPSC-derived neurons and astrocytes can develop endogenously and build fast and robust functional phenotypes. Second, we described their further developmental potential by optimized cell culture environment. Third, we proved that these reductionist human neuronal networks can decipher mechanistic drug effects and serve as potential tools for drug efficacy evaluation. Hopefully, our findings will bring the field closer to translatable neurological and neuropsychiatric modeling and treatment. Since many, if not most, neurological and psychiatric disorders involve pathophysiology that is related to changes in synaptic function and neuronal network activity (Forrest et al., 2018), our approach could serve as a fast way to an effective human-relevant model system to decipher these functional changes and their responses to pharmacological rescue approaches. Considering the trend in the stem cell field to focus on structural aspects of various neuro-pathologies when modeling diseases, we encourage the field to put more efforts in modeling functional aspects of these pathologies. At the end of the day, it is not the structure, but rather the function that matters.

7 FUTURE PERSPECTIVES

While we have moved a couple of stones to improve the model system and test it in a context of drug evaluation, there are still many un-walked paths that remain to be explored in the future.

First, even though the major focus of this work was related to functional assessment of neurons and networks, the work in this thesis was limited to neuronal spiking and bursting as measurable read-out to assess neuronal network function. Detected spiking and bursting activity are local field potentials in the frequency band of >1000 Hz. Another functional readout which is arguably as relevant as spiking activity, is the assessment of low frequency local field potentials (0.1 towards 500 Hz). Low frequency local field potentials can be recorded *in vivo* by implanted electrodes or electroencephalography, thus their assessment in *in vitro* preparations might represent a potential clinical relevance. Future assessment of low frequency oscillations including delta, theta and gamma oscillations and their development in our model system will be of a great interest.

Second, while the MEA system we used in this study still does the job, upgrade to multi-well systems (24 to 96-well format) or higher electrode resolution (HD-MEAs, >1024 towards 25,000 electrodes) should be considered for the future. Optimization and upscaling to multi-well systems would be a prerequisite for future higher throughput drug evaluation studies. Further, high-spatial resolution by high-density MEAs could decipher characteristics of network dynamics and modulations in more detail and could successfully address important subsampling effects occurring in systems with smaller numbers of electrodes. Additionally, the combination of MEA technology with currently growing optical readouts such as voltage imaging using voltage sensitive dyes could offer better resolution on single cell contributions to the recorded signal.

Third, the combination of the MEA platform with optogenetic tools will be extremely valuable to resolve cell-type specific mechanistic effects on development and therapeutical implications.

Fourth, we proved that the application of healthy bio-samples, such as healthy human CSF has an impact on neuronal network activity and maturation. The next step would be to test pathological bio-samples, such as CSF samples derived from patients with Alzheimer's or other neurodegenerative diseases.

Last, it is important to keep in mind that even though this model is human-specific, it is still a model. Observations we make in a model might not be valid in another model or in patients. Thus, generalization of any result should be made with caution. Modeling with iPSC-derived cells should be combined with animal models to test systemic aspects.

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APPENDIX

Article I

Article II

Article III

Article IV