Master Thesis

Engineering solutions to neural stem cell differentiation challenges

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Abstract

Background Damage dealt to the central nervous system (CNS) caused by trauma or disease can have detrimental effects on human quality of life because the CNS has limited regenerative capabilities. Efforts to replace lost neural cells require improved knowledge and methods for differentiation of neural stem cells (NSCs).

Objective In this thesis, I aim to chart our current scientific knowledge and progression of neural differentiation and explore practically the feasibility of continuous ultrasound (US) stimulation on neural progenitor cells (NPCs) differentiating into neurons *in vitro*.

Methods I performed a literature study examining previous studies that investigated electrical stimulation, nanoparticles, or ultrasound to improve *in vitro* or *in vivo* differentiation of neural stem cells. Using finite element method (FEM) frequency analyses with COMSOL Multiphysics, I investigated the use of a 24-wells plate with piezoelectric lead zirconate titanate $[Pb(Zr_xTi_{1-x})O_3]$ (PZT) US transducers. Additionally, I validated *in vitro* previous findings on the feasibility of differentiation of NSCs to NPCs.

Results With the knowledge gained from literature and findings from the experiments, I created a mold for the fabrication of a custom variant of a 24-wells plate made with polydimethylsiloxane (PDMS) to which 2.03 mm thick PZT can be mounted.

Conclusion Future research efforts should focus on further developing this technique, specifically into electrical schemes to optimize US transmission to NPCs.

Contents

Part I

Literature Study

Chapter 1

Literature Introduction

1.1 Problem

The central nervous system (CNS) of humans has limited capabilities of self-repair or regeneration. Therefore, degeneration of neurons due to illness or neural damage caused by trauma is often effectively irreversible [1]. Once the damage is done, recovery requires replacing neurons that have degenerated.

Replacing degenerated neurons can be done in two ways. On the one hand, one can use a patient's cells. These cells are referred to as *endogenous* cells. On the other hand, cells can be obtained from an external source. These are *exogenous* cells.

Endogenous methods can be divided into two subtypes. Firstly, a method where cells are forced to convert into neurons in the afflicted area. Usually, glia cells are the target of this process. Secondly, a method where cells are recruited from neurogenic niches. While research showed that hippocampal neural stem cells (NSCs) actively differentiate into neurons in rats, the same has not yet been proven in humans and is, in fact, disputed [2, 3]. Therefore, the viability of this method as therapeutic is doubtful. Thus far, endogenous methods have not produced lasting results *in vivo*.

Exogenous methods have shown more promising results. Cell survival, differentiation, and even behavioral change, specifically locomotor improvement in spinal cord injury (SCI), have been observed in animal models [4]. Exogenous methods rely on cultured neurons.

1.2 Knowledge gap

Our understanding of neurons' development and interplay in healthy and pathological settings is limited. Research into their properties and role in the brain often requires animal models.

Animal models are of limited aid in this process because of the following. Firstly, studying neurons retrieved from animal models *in vitro* requires harvesting neurons which, although

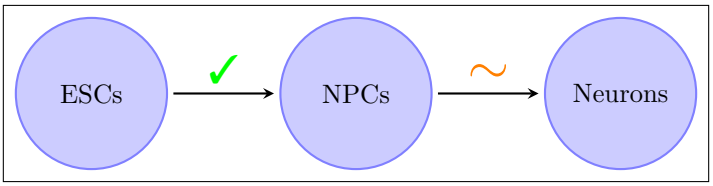

Figure 1.1: Problem statement. Differentiating embryonic stem cells (ESCs) to neural progenitor cells (NPCs) is relatively well understood. Differentiating neural progenitor cells (NPCs) to neurons still poses challenges.

possible, is an involved and complicated process with low yields [5]. Secondly, reducing the number of animal models while maintaining the same quality research is preferable.

An alternative method of obtaining neurons is to let embryonic stem cells (ESCs) differentiate into neural progenitor cells (NPCs) and then into neurons, as illustrated in **Figure 1.1**. The first step in this process is well-researched and practically achievable with high yields [6, 7]. The second is more complicated. Yields of this process are low, and the process leads to a heterogeneous group of neurons [8].

Various solutions have been proposed to this problem. Exposing developing cells to electrical stimulation (ES), nanoparticles (NPs) or ultrasound (US) and growing cells on various structures and with a multitude of growth factors has been attempted. However, our knowledge of why the factors mentioned have the examined effects on differentiation is yet to be cleared. In their respective chapters, I dive deeper into studies to understand and improve the differentiation of NSCs.

1.3 Structure

In this thesis, I will explore the use of US to improve differentiation of NPCs to neurons.

Part I describes the advances in techniques invented and described in literature. Specifically, Part I will explore strategies with electrical stimulation in **Chapter 2**, nanoparticles in **Chapter 3**, and ultrasound in **Chapter 4**.

Then, Part II explores the practical aspects of developing an US system that can stimulate NPCs. First, **Chapter 6** provides an overview of my approach. Then, **Chapter 7** describes the work I did to develop a model for the US transducer, while **Chapter 8** sets out the details of the cell culture work I did in an exploration of the possibilities.

Chapter 2

Effects of electrical stimulation on differentiation

2.1 Mechanism of action

Here, I will describe the established mechanisms, as well as dive into newer ventures into understanding the mechanisms of how ES can result in neuronal differentiation. The mechanisms by which ES affects cells are under ongoing investigation. Many have thus far been identified, but it remains unclear if any one of those is essential and sufficient, or that perhaps multiple mechanisms are required to elicit effects. Because of the non-specific nature of electrical stimulation, it is reasonable to expect multiple downstream effects via various pathways. Some of the pathways that have been exposed thus far depend on Ca^{2+}, while others are independent.

2.1.1 EF and EMF

Generally, two types of ES are distinguished: electric fields (EFs) and electromagnetic fields (EMFs) [10]. EF generally is generated externally and transmitted via electrodes. The resulting electrical current passes the exposed material directly. EMF is also an electrically based stimulus. However, in EMF, the magnetic component of the stimulus stimulates tissue. Generally, EMF is used in non-invasive procedures, such as transcranial stimulation.

2.1.2 Ca^{2+}-independent

When triggered by ES, epidermal growth factor receptor (EGFR) leads to the activation of MAPK, the key component of the MAPK pathway. In **Figure 2.1**, I illustrated this effect and the ones described later for reference. Activation of several types of receptor tyrosine kinases (RTKs) has a similar effect. In addition, RTKs directly contributes to the MAPK

Background

MAPK pathway
The MAPK pathway, or Ras-Raf-MEK-ERK pathway, refers to a group of proteins that interact upon activation of a receptor and leads to changes in processes in all mammalian cells. These include cell division, differentiation, and cell survival and -death.

NMDAR/Rac1/actin pathway
The NMDAR/Rac1/actin pathway refers to a neuron-specific signalling pathway that is involved in the development of neurons via, among others brain-derived neurotrophic factor (BDNF).

Rho pathway
The Rho signalling pathway plays a crucial role in cytoskeletal reorganization [9]. Downstream effectors of the pathway are mDia and Rho-associated coiled-coil forming kinase (ROCK). mDia catalyses actin polymerization. ROCK inactivates myosin phosphatase by way of phosphorylation, thereby increasing myosin.

PI3K pathway
The phosphoinositide 3-kinase (PI3K), or PI3K/AKT/mTOR pathway regulates the cell cycle. One of the effectors of the pathway is AKT which, among many effects, activates cAMP response element-binding protein (CREB) downstream.

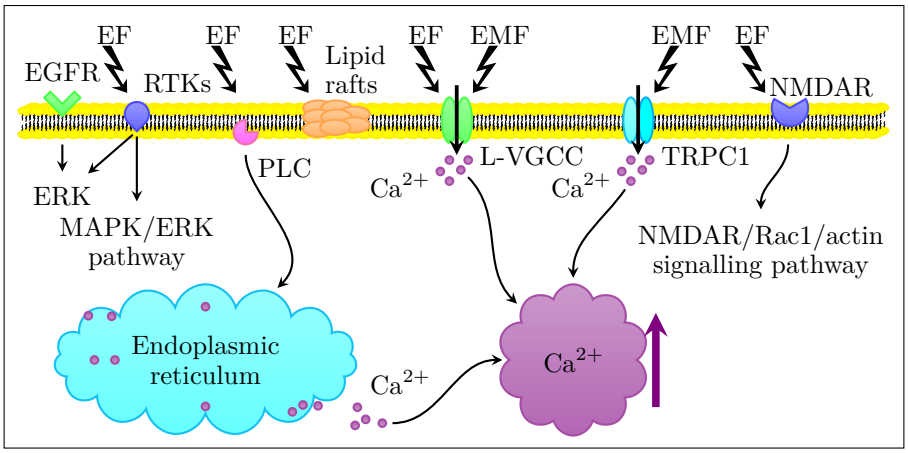

Figure 2.1: Mechanism of action of the effects of electrical stimulation on Ca^{2+} influx in NSCs and other pathways. Electric field (EF), electromagnetic field (EMF), epidermal growth factor receptor (EGFR), receptor tyrosine kinase (RTK), *N*-methyl-D-aspartate receptor (NMDAR), phospholipase C (PLC), L-type voltage-gated Ca^{2+} channel (L-VGCC), transient receptor potential canonical 1 (TRPC1). Adapted from [10].

pathway. *N*-methyl-D-aspartate receptors (NMDARs), shown to be activated in an *in vitro* rat model [11], might also play an important role. Interactions between NMDAR, actin filaments, and Rac1 cause activation of the aptly named NMDAR/Rac1/actin pathway. This pathway is involved in actin polymerization, specifically in dendrite growth [12]. Further, EF stimulation can lead to aggregation of glycolipids present on the membrane. These aggregations are referred to as lipid rafts. Lin *et al.* [13] showed that membranes containing lipid rafts are responsible for EF sensing and triggering the Rho- and PI3K pathway.

2.1.3 Ca^{2+}-dependent

Alternatively, ES results in increased levels of Ca^{2+}. Many mechanisms cause increased Ca^{2+} levels. For example, both EF and EMF affect L-type voltage-gated Ca^{2+} channel (L-VGCC) and transient receptor potential canonical 1 (TRPC1), the activation of which triggers increased Ca^{2+} levels. Phospholipase C (PLC) also has been shown to be affected by low EF [14]. They respond to activation by inducing the endoplasmic reticulum (ER) to release Ca^{2+}.

Increased levels of Ca^{2+} affects many pathways in the cell involved in cell growth and differentiation. An overview of our current understanding of the downstream effect of Ca^{2+} influx is shown in **Figure 2.2**. The MAPK pathway is modulated by Ca^{2+} increase [15]. In neurons, specifically, PYK2, a Ca^{2+}-dependent kinase, induces Ras activation by recruiting the Grb2-SOS complex to the membrane, which in turn affects ATP, L-glutamatic acid, acetylcholine, dopamine, serotonin and GABA concentrations [10]. Further, an increase of Ca^{2+} leads to phosphorylation of CREB, a transcription factor. CREB, when activated recruits CREB binding protein (CBP). This complex can then affect the expression of BDNF. BDNF is crucial for development of neurons [16].

2.2 Electric field

Adipose-derived stem cells (ADSCs) are also able to differentiate to NPCs. This cell type, however, is generally limited in terms of further differentiation to neurons. Heo *et al.* [17] looked to alleviate this issue by incorporating an electrically conductive hydrogel as a basis for the cells to grow on. The hydrogel was created by mixing polyethylene glycol (PEG) with poly(3,4-ethylenedioxythiophene):polystyrene sulfonate (PEDOT:PSS). PEG is widely used to create microwells, while PEDOT:PSS is a conductive polymer with known chemical

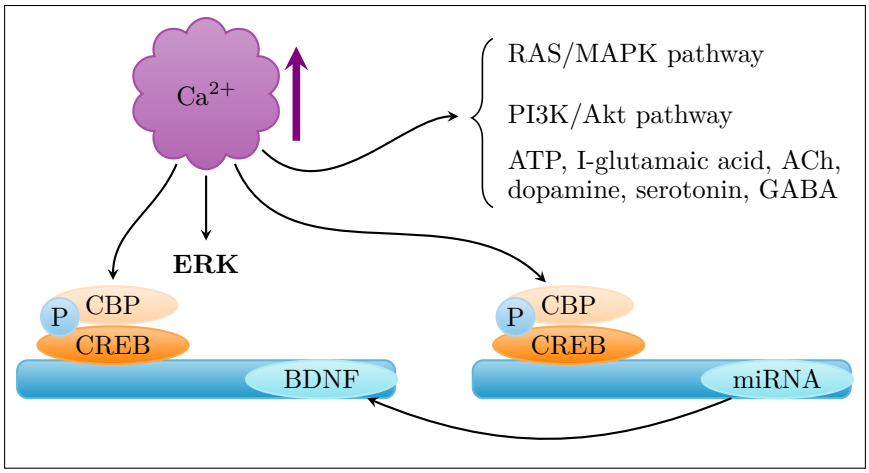

Figure 2.2: Mechanism of action of the downstream effects of Ca^{2+} influx in NSCs. cAMP response element-binding protein (CREB), CREB binding protein (CBP), brain-derived neurotrophic factor (BDNF), acetylcholine (ACh), extracellular signal-regulated kinase (ERK). Adapted from Zhu *et al.* [10].

stability and biocompatibility. The authors used this material to create microwells. Exposing this substance to UV light allows it to form cross-links. Neurogenin 2 (Ngn2), neurogenic differentiation 1 (NeuroD1), neuron specific enolase (NSE), and TAU were studied compared to the housekeeping gene GAPDH in a study at the mRNA level. When differentiating human ADSCs on the mixed PEG/PEDOT:PSS while applying ES, several neuronal differentiation markers are significantly increased compared to nonconductive wells. In **Table 2.1**, I provide an overview of the experiments discussed here.

Where Heo *et al.* [17] used PEDOT:PSS, Fu *et al.* [20] used an alternate electrically conductive material; graphene oxide (GO). Combining this material with poly(L-lactic-co-glycolic acid) (PLGA) created a conductive composite membrane on which murine NSCs were planted. Here, the authors investigated neurite growth as a marker for neural differentiating using a confocal laser scanning microscope. The ES was achieved using two platinum electrodes placed in a 24-well plate, exposing the cells to 100 mV for 1 h each day with an electrical frequency 100 Hz. Cells grown on PLGA/GO with ES had significantly longer

Table 2.1: Overview of experiments.

authors	source	stem cell type	research	experiment
Du *et al.* [18]	human	neural crest	*in vitro*	stimulation with different amplitudes / potential gradients of pulsatile ES
Du *et al.* [18]	rat	neural crest	*in vivo*	stimulation with different amplitudes / potential gradients of pulsatile ES
Meng *et al.* [19]	mouse	NPCs and NSCs	*in vitro*	stimulation with an 250 mV mm^{-1} EF and bFGF.
Heo *et al.* [17]	human	ADSCs	*in vitro*	stimulation with unspecified ES in conductive PEG/PEDOT:PSS microwells.
Fu *et al.* [20]	mouse	NSC	*in vitro*	stimulation with 100 mV even, rectangular pulses at 100 Hz.

neurites than PLGA without ES controls. Interestingly, they showed that cells grown with PLGA/GO without ES and cells grown with PLGA with ES both also significantly showed longer neurites that controls, but shorter than PLGA/GO with ES. These results point to separate mechanisms for GO and ES, which synergize when used together.

Electrical stimulation can also be combined with traditional growth factors to stimulate differentiation from NSCs to neurons. Using traditional growth factors in combination with electrical stimulation can also show a synergistic effect Meng *et al.* [19]. When bFGF and EF $150\,\mathrm{mV\,mm^{-1}}$ are combined on a 3D scaffold, cells showed morphological and functional similarities to neural tissue. For instance, branching of neurites, cell count, and development of neuronal networks were all similar. Furthermore, the authors demonstrated no discernible difference in survival of NSCs when exposed to EF compared to controls.

2.3 Electromagnetic field

Another study investigated neurospheres [21]. Neurospheres are suspended 3D spherical structures formed by cultured NPCs and were first described by Reynolds & Weiss [22]. They showed that this structure contained a heterogeneous collection of NSCs and NPCs by re-plating single cells of this structure and examining the potency to form secondary neurospheres. The fraction of NPCs is generally higher than NSCs. To be classified as neurospheres, spheres must have a diameter of $80\,\mu\mathrm{m}$. The researchers observed that only a small subset of all cells could initiate neurospheres, thereby demonstrating these cells' multi-potent and self-renewal capabilities. A biological equivalent of neurospheres is poorly understood as they appear to develop differently *in vivo* than they do *in vitro*. Nonetheless, because of their composition of largely NPCs, they are a helpful tool when studying neural differentiation.

Sefton *et al.* [21] used an EMF of $250\,\mathrm{mV\,mm^{-1}}$. They found the neurospheres, constructed from neural cells retrieved from adult mouse brains, to be more proliferated and more neuron-like than controls that received no EMF.

Chapter 3

How nanoparticles affect differentiation

3.1 Introduction to nanoparticles

Another research angle focuses on introducing small particles to stimulate neural stem cells to differentiate.

These nanoscale particles, or NPs, can be made from various materials. Generally, they are grouped as *organic*, *inorganic*, and *hybrid* NPs based on their composition. Organic materials include liposomes, micelles, and small polymers, among others, while inorganic materials include fullerene (a spherical form of graphene), gold, and silica, among others [23]. Hybrids NPs use a structure of core and shell that contain materials belonging to the other groups, for example, a metal core coated with a polymer shell.

3.2 Mechanisms of uptake

For NPs to affect cell function and differentiation, they must interface with cells. The primary method in which this occurs is endocytosis. After endocytosis, the specific materials that the NPs is made up of determines the effect the NPs have on the cell. Endocytosis of NPs can, depending on the materials and size of the particles, occur via different methods: *receptor mediated endocytosis*, *phagocytosis*, *pinocytosis* and *caveolae*. Generally, this process results in the NPs being present in an early endosome.

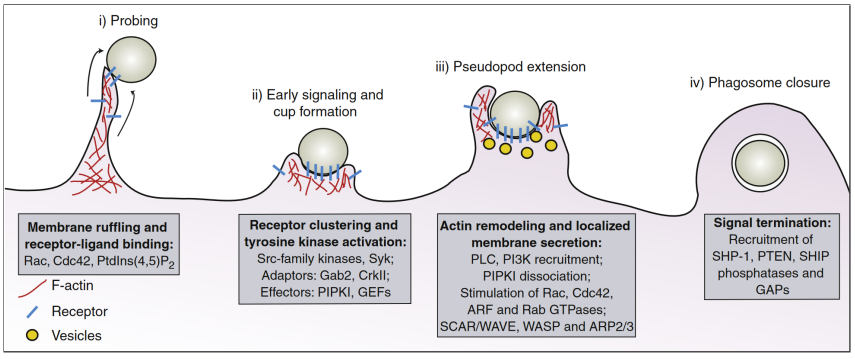

Figure 3.1: Mechanism of action of phagocytosis [24].

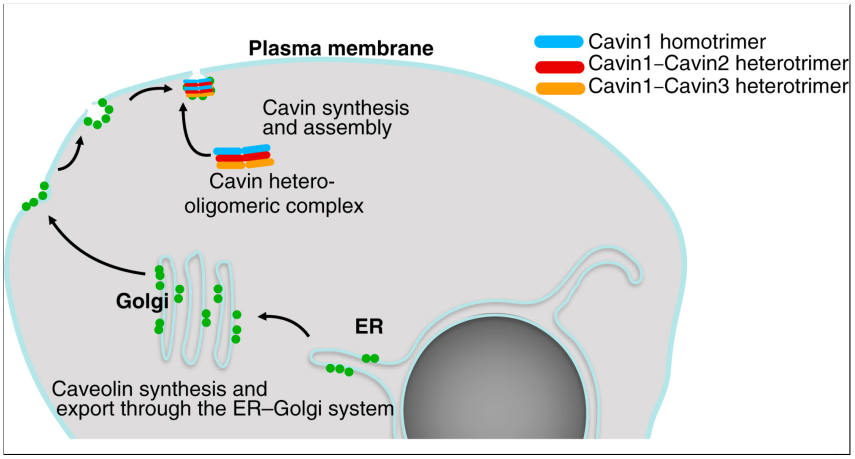

Figure 3.2: Mechanism of action of caveolae. Adapted from Parton *et al.* [29].

3.2.1 Receptor Mediated Endocytosis

Receptor mediated endocytosis (RME) is the process of endocytosis that selectively engulfs particles that bind to specific receptors on the cellular membrane [25]. Often, viruses use this mechanism to enter a cell, but NPs of appropriate size also utilize it. This process is mediated by clathrin and highly dependent on size. The optimal size of particles, as shown experimentally by [26], is around 50 nm. Upon docking, or binding of clathrin receptors, particles are taken up into vesicles which combine and merge into endosomes.

3.2.2 Phagocytosis

Phagocytosis, which loosely translates to *cell that eats*, is another method of ingesting substances into a cell body. Almost all cell types are, in some capacity, capable of phagocytosis. Notably, cells performing this action with high efficiency are mostly immune cells, for example, neutrophils, macrophages, and dendritic cells [27]. However, phagocytosis plays a crucial role in tissue homeostasis. Phagocytosis usually takes up relatively large particles 0.5 µm.

The mechanism can be categorized into four stages (**Figure 3.1**). Firstly, a cell membrane probes the extracellular space by extending the membrane. The extensions contain a high concentration of phagocytic receptors. Then, if they encounter a particle that binds to the receptors sufficiently, the bound particle starts to be surrounded by the membrane caused by actin remodeling. This is triggered by the recruitment of protein- and lipid kinases due to signaling pathways triggered by the phagocytic receptors. Thirdly, pseudopods form that encircle the particle and extend the membrane [24]. Because clathrin is usually not involved in the process, this process is referred to as clathrin-independent endocytosis [23, 28]. Finally, the membrane attaches to itself, fully engulfing the particle and forming a phagosome [24].

3.2.3 Pinocytosis

Pinocytosis, which, similar to phagocytosis, loosely translates to *cell that drinks*, is a subset of endocytosis that is employed to absorb fluids and smaller particles dissolved therein into cells non-specifically. As in phagocytosis, the result of this process is a phagosome.

3.2.4 Caveolar endocytosis

Caveolar endocytosis, consisting of caveolae, is another type of endocytosis that can be used to uptake NP. Membrane proteins of the caveolin-family initiate caveolae formation. Caveolin-1 plays this role in non-muscle cells, while caveolin-3 does so in muscle cells. While these proteins start forming a pit in the membrane, Cavin-trimers assemble. Next, the trimers associate with the caveolin proteins to form complex structures that pinch off the membrane and form carriers that become early endosomes.

3.3 Some approaches

3.3.1 Ferritin

Ferritin-coated NPs have been used to help stimulate NSC differentiation. Ferritin is a naturally occurring protein with a size between 8 and 12 nm. Ferritin used in this experiment was harvested from equine spleens. Ferritin NPs can be internalized via RME [30]. The researchers continuously added ferritin NPs into neurospheres during the development of NSCs. They then investigated the differentiation of these spheres into NPCs. They found no difference in early neurosphere formation. However, without ferritin NPs, neurospheres merged to form larger neurospheres making for heterogeneously distributed sizes. Neurospheres treated with ferritin also exhibited significantly more differentiation capacity compared to controls, evidenced by histochemical analysis and qPCR results.

3.3.2 Valproic acid labeled chitosan

Others have combined two previous discoveries [31]. Valproic acid (VA) and chitosan nanoparticles (CN) were both shown to help recovery after SCI. Using VA labeled CN (VA-CN) after inducing SCI in rats allowed better recovery compared to controls that received no treatment, VA only or CN only, evidenced by increased Basso, Beattie and Bresnahan (BBB)-scores. BBB-scores are a metric for evaluating motor function after SCI [32]. Moreover, they showed that this result is likely due to increased differentiation of NSCs. The authors used Tuj-1 as an immunohistochemical marker for differentiation in NSCs four weeks after surgery. They showed that VA-CN, but not VA nor CN had significantly increased Tuj-1 levels compared to no treatment [31]. The authors did not investigate the uptake method of NPs, nor the mechanism of action.

3.3.3 Gold

Zhang *et al.* [33] also combined two approaches. They coated gold NPs with synthetic glycosaminoglycans (GAGs) with added glyco and sulfonate functional units designed to optimize the related signaling pathway. GAG triggers the previously mentioned MAPK pathway [34]. Namely, GAG can bind fibroblast growth factor 2 (FGF2), which in turn activates mitogen-activated protein kinase (MAPK). The choice of synthetic GAGs arises from the limited applicability of large, naturally occurring GAGs. Because of their size, they exhibit low purity. Synthetic GAGs can be optimized by tweaking the ratio of functional units for better activation of the related pathway.

They took advantage of the photothermal properties of gold by irradiating gold-NPs with a laser for 10 s. This achieves activation of a second pathway, one related to RA. They investigated this by measuring the amount of extracellular RA. They saw a significant decrease when comparing directly before and after radiation, but only in the presence of gold-NP coated with synthetic GAG. This combined approach improved neural differentiation of ESCs and increased the purity of differentiated neural cells.

Background

RA pathway

The RA pathway is involved in a variety of biological functions. retinoic acid (RA) is the main metabolite of vitamin A. Upon binding its nuclear receptors retinoic acid receptor and retinoic X receptor it regulates gene transcription, influencing differentiation, proliferation and development [35].

Chapter 4

Understanding the mechanisms of ultrasound and differentiation

4.1 Introduction

US refers to sound with frequencies inaudible to humans ($> 20\,\text{kHz}$). Although, in essence, US is no different from ordinary sound, the effects of it on tissue and cells US are vastly different. Further, the frequency and pressure that characterize the sound waves influences how US affects tissue. Ultrasound with high intensity, for example, has been used for tumor treatment as a means of non-invasive thermal ablation [37]. For this, US is focused on volumes with precision in the order of millimeters.

US has also been shown to be able to modulate neuronal activity. To achieve this, US with lower intensities is used. The mechanism(s) of action by which the resulting modulation is achieved is, to date, poorly understood. Neuromodulation, as it is commonly referred to, can be achieved with a plethora of techniques, including electrical, electromagnetic, drug-induced, or ultrasonic stimulation. The goal of neuromodulation is to change the neural activity of a specific neuron or group of neurons to resolve or reduce some ailment. Neuromodulation has shown applications for pain relief [38], drug-resistant epilepsy [39], and more [40].

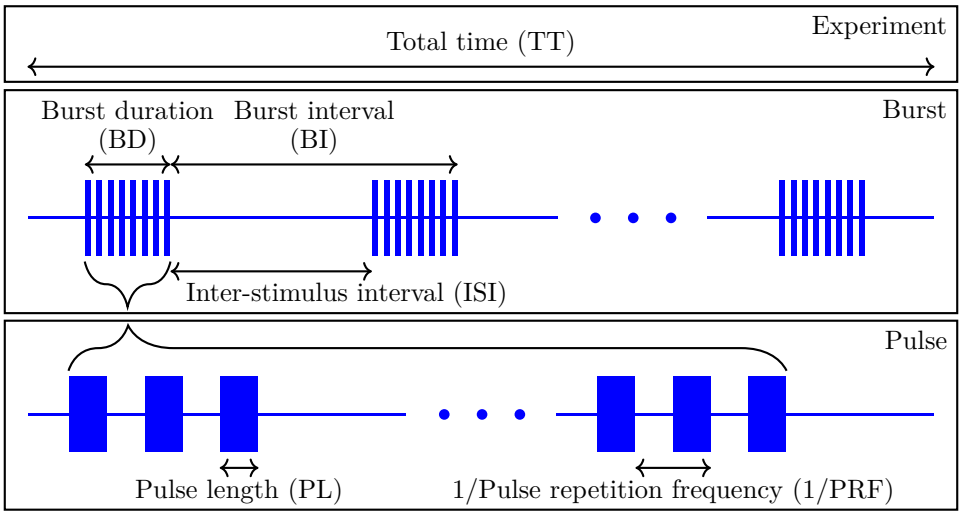

Figure 4.1: Schematic representation of commonly used US wave properties. Adapted from [36].

4.1.1 Safety metrics

If ablation of tumors is possible using US, naturally, safety concerns are expressed for using US on healthy neurons. Tissue damage has occasionally been reported with some applications of US. However, many showed that the intensity parameters could be adjusted such that no damage is visible [36]. Three metrics commonly express the safety levels of US. Those being *intensity*, *thermal index (TI)* and *mechanical index (MI)*.

Intensity Intensity has three commonly used variants associated with it: spatial-peak, pulse-averaged (I_{SPPA}), spatial-peak, burst-averaged (I_{SPBA}), and spatial-peak, temporal-averaged (I_{SPTA}). Understanding these metrics requires an understanding of frequency (f) and sound pressure (p) of sound. f, expressed in Hz, is a measure of how often a sound wave oscillates per second, while p is the deviation from ambient pressure caused by a sound wave. Intensity, often measured in $\mathrm{W\,cm^{-2}}$, is then determined by:

$$I = vp, \tag{4.1}$$

with v the velocity of the particle that causes the sound pressure.

The three intensity variants now come from the location, and characteristics v and p are measured. Spatial-peak refers to the intensity at that point in space that has the highest intensity. This will be located in the middle of the US beam. As illustrated in **Figure 4.1**, a pulse is typically not provided continuously but in bursts. Therefore, pulse-averaged intensity takes the average intensity of the pulse length (PL), and burst-averaged intensity takes into account the intensity during the burst duration (BD). At the same time, the temporal-average considers the total time (TT).

Thermal index TI is a ratio of two types of sound power. Sound power (W_p) is sound intensity integrated over the surface it is subjected to, or:

$$W_p = \int_A I \, dA, \tag{4.2}$$

where A is the area subjected to the sound beam.

TI follows then as:

$$TI = \frac{W_p}{W_{deg}} \tag{4.3}$$

where W_{deg} is sound power needed to heat up the tissue by 1 K [36].

Mechanical index MI was developed in an attempt to assess the risk of microbubbles (MBs) induced cavitation (more on this subjects in **Section 4.2.2**). It is calculated as the ratio of the peak negative pressure (PNP) and the square root of the frequency used, or:

$$MI = \frac{PNP}{\sqrt{f}}. \tag{4.4}$$

This ratio was consistent with the probability of cavitation-induced damage to tissue [41].

4.1.2 Typical setup

To better understand the experiments, I will discuss further in this thesis a short overview of what a typical US set up for research purposes is in order. An example is visualized in **Figure 4.2**. Firstly, an US transducer is required. This device receives electrical input

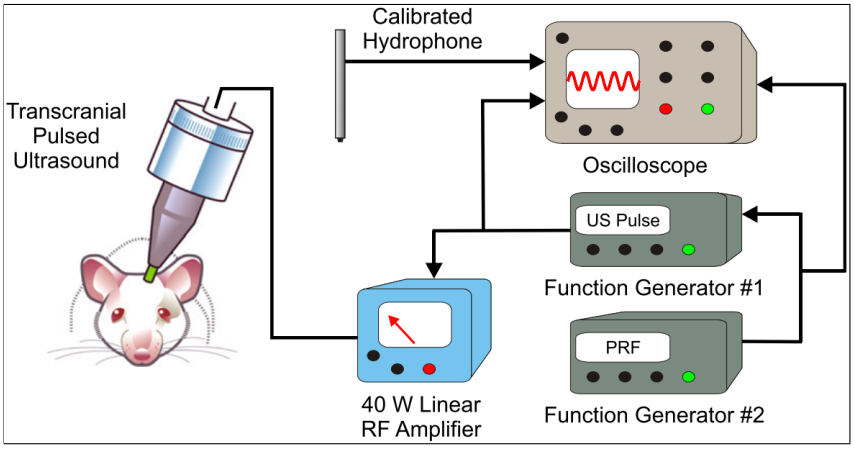

Figure 4.2: An example of an US setup for neuromodulation research. In this experiment, the brain of mice was stimulated through the skull [42].

and converts it into an US wave using piezoelectric elements [43]. The electric inputs are generated by one or more function generators. The desired output wave is configured on these devices. Finally, the electrical signals can be visualized by an oscilloscope. A linear amplifier is placed between the function generators and the US transducer to scale the electrical signal, guaranteeing that the signal is in the range that the transducer can receive. Finally, a hydrophone can be used to directly measure the US that the object was exposed to.

4.1.3 Outline

In this thesis, I will discuss ultrasonic neuromodulation only. In **Section 4.2**, I will review the progress that has been made in unraveling the mechanism of action of US sensitivity of neurons. I will explore nonthermal effects such as cavitation, membrane oscillation, or radiation forces, as well as thermal effects such as via temperature-sensitive channels or effects arising from the varying rate of temperature change.

In some applications, it is desirable to target individual neurons. For that purpose, however, focused ultrasound is not precise enough, with axons and dendrites having a diameter of about 1 µm. Even though it is not fully understood how US affects cells precisely, researchers have attempted to alleviate some of the problems that diminish the usefulness of US-based treatments from being widely adopted. One of these efforts is a new area of research proposed by Ibsen *et al.* [44] called sonogenetics. In **Section 4.3**, I will discuss the advances in the field and compare the technique to optogenetics.

Finally, in **Section 5.3**, I summarize the findings in the previous chapters and look at what future work will propel the field forward.

4.2 Intrinsic ultrasound sensitivity

For roughly a century, it has been known that high-frequency sound waves can have a stimulatory effect on cells [45]. Numerous articles have attempted to explain mechanisms by which these sound waves, now called US, might modulate excitable cells. Here, I will describe mechanisms that can (partially) explain intrinsic US sensitivity. Because of the mechanical nature of acUS, a reasonable assumption might be that thermal effects play a role in transferring mechanical energy to electrical energy in the cell. I will therefore describe thermal effects separate from nonthermal effects.

4.2.1 Thermal effects

Sensing changes in temperature is crucial for the survival of most living organisms. Unsurprisingly, a large family of molecular sensors known as transient receptor potential (TRP) has evolved [46]. These ion channels can mediate all kinds of sensory information including, but not limited to, visual, auditory and thermal. Focusing on the thermal variants thermo-TRP (T-TRP), they are found in the peripheral nervous system (PNS) [47]. If activated, they generate a signal which propagates to the CNS, where they are interpreted as temperature. Knowledge of their activation mechanism is important to explore if they can also play a role in US sensitivity. T-TRPs are channel-forming proteins with high affinity for Ca^{2+} cations. This allows them to depolarize the sensory neurons in the PNS and trigger action potentials. Interestingly, the expression of orthologous proteins in different species does not lead to sensitivity for the same trigger. TRPA1, for example, is temperature-insensitive in zebrafish but acts as a cold receptor in rodents [47].

Technically, every molecule can be considered sensitive to temperature because a higher temperature, in essence, means that molecules vibrate more. The difference between most other ion channels and T-TRPs is their Q_{10} value. This is a measure of how much the reaction rate of a channel changes when the temperature increases by 10°. While a regular ion channel has a Q_{10} of around 3, the Q_{10} of TRPV1 is around 50 [47]. Some kinetics of T-TRPs are modulated allosterically by Ca^{2+}. The Q_{10} for a protein is found via:

$$Q_{10} = (I_1/I_2)^{10/T_2 - T_1} \tag{4.5}$$

where current amplitudes I_1 and I_2 are measured at temperatures T_1 and T_2.

The high Q_{10} value of T-TRPs indicates that they are highly sensitive to small changes in temperature, which means they should be considered as a potential mechanism of action through which US can operate.

Other work indicates that cells subjected to an infrared stimulus, which increases temperature, respond depending on the rate of temperature rise [48]. Because this phenomenon is not directly expected from T-TRPs, this might point to yet another mechanism at work.

Notably, both studies discussed here use alternative means to induce heat and might not be phenomena occurring during US modulation.

4.2.2 Nonthermal effects

Cavitation

Several research groups attempted to develop a unified model of how US affects biological tissue. For example, Krasovitski *et al.* [49] proposes that the lipid bilayer of cells plays an important role, acting as a bilayer sonophore (BLS). This term indicates that the bonds between the monolayers of the membrane are (temporarily) broken during exposure to US, creating oscillating monolayers (**Figure 4.3**). With higher input energy, larger cavitations or membrane ruptures can occur in this model, per observations in goldfish membranes. Initially, the resulting tension in the membrane can trigger mechanosensitive proteins, whereas, in further stages, membrane proteins or membranes can be irreversibly damaged.

Membrane capacitance

Prieto *et al.* [50] investigated the effect of the membrane on a membrane model without proteins. Using low-intensity US on the lipid bilayers, they claim that radiation force, rather than cavitation explains the biological response. Radiation force is a phenomenon resulting from US encountering an object (the membrane, here):

$$|F| = \frac{2\alpha I}{c} \tag{4.6}$$

where $|F|$ is the magnitude of the radiation force, α is a coefficient related to absorption of energy of the object, I is the temporal average intensity at the location of the measurement and c is the speed of sound in the medium.

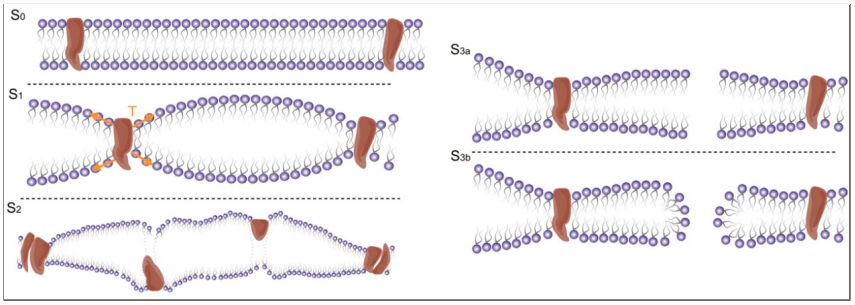

Figure 4.3: Bilayer sonophore model. S_0 shows regular membrane. S_1 depicts cavitation of the BLS caused by increased tension. S_2 shows the state where growing tension lead to damaged membrane proteins. Continued exposure to this state can lead to rupture (S_{3a}) or pore forming (S_{3b}). Adapted from Krasovitski *et al.* [49].

Where Krasovitski *et al.* [49] points to proteins in the membrane as the main contribution to US stimulation, [50] shows that the capacitance of the membrane changes dynamically before settling in a steady state. The dynamic effects related to the start of stimulation might explain why stimulation of equal length but different activation patterns show different responses. Although this experiment was done *in vitro*, the results suggest that *in vivo*, too, radiation force may play an essential role in neuronal sensitivity to US.

Thermal versus nonthermal

To investigate if thermal or nonthermal forces lay at the foundation of US sensitivity, Kubanek *et al.* [51] investigated the behavior of *C. elegans*. They compared animals unable to sense small temperature fluctuations to wild type. US stimulation resulted in similar behavior between groups, showing that small temperature changes in the animal model are not required for US response. In addition, they showed that animals with a specific *mec-3* mutation fail to show the same response to US stimulation. *mec-3* is known to be involved in gentle and harsh touch sensation. They checked if specifically US caused the shown effect by comparing all actual stimulation to a sham stimulus. This sham stimulus appears the same as an actual stimulus but does not carry energy and thus cannot stimulate.

Interestingly, Ibsen *et al.* [44] reported that TRP-4 acts as a mechanosensitive ion channel when triggered by MBs, which are gas-filled monolayers that can amplify US locally via cavitation. Kubanek *et al.* [51] showed that when not triggered by MBs, rather directly with US, it no longer acts differently compared to wild type, but these findings were not reproducible by Kubanek *et al.* [51]. Their findings suggest that the differences found by Ibsen *et al.* [44] were caused by other variations in the model.

Notch-signalling pathway

Recently, Wu *et al.* [52] clarified some of the long-term effects that US exposure has on neurons. They noted that low-intensity pulsed US on neural rat stem cells might be regulating proliferation and differentiation through the Notch-signalling pathway. This insight suggests that low-intensity pulsed US could be an attractive target for a therapeutic means to restore innervation, such as spinal cord injury. Also, since the Notch-signalling pathway is heavily involved in embryonic development, developmental biological problems might be targets for further research.

Concluding

All in all, the intricacies of US modulation are still not fully understood. Groups have shown convincing arguments for cavitation [49], membrane capacitance [50] or thermal effects [46–48] as the primary contributor to US sensitivity. At the same time, also long-term effects have been shown [52]. These results show that while many different aspects are being identified that US affects, we do not fully grasp the intricate details of US modulation.

4.3 Genetically induced ultrasound sensitivity

So far, I've discussed intrinsic US sensitivity properties and their mechanisms, but cells can also be sensitized to US. The research field of sonogenetics emerged after the discovery that neurons of *Caenorhabditis elegans* can be genetically modified to become sensitive to low-pressure US by Ibsen *et al.* [44]. Sonogenetics can be seen as a counterpart to the, by now, commonplace optogenetics. Where optogenetics operates through genetically modified cells becoming sensitive to visible light, sonogenetics operates using low-pressure US. The main advantage of US stimulation over light-based stimulation is that US can be applied non-invasively [44]. This approach has, in theory, a couple of crucial advantages. Firstly, it removes the necessity of surgery to attach an optical fiber to the cranium of animals.

During surgery, animals are exposed to high infection risks; afterward, the animals can suffer from permanent brain damage. Also, the animals have to recover, which takes valuable time (around two weeks [53]). Secondly, the presence of the implant during behavioral testing can influence neural activity and, by extension, the natural behavior of the animal [53]. Naturally, efforts are put into making implants with less invasive optical fibers, leading to impressive results [53], but current US solutions are already non-invasive. Because US can be targeted without being attached to a test animal, the animals have no implants on their heads. This makes the cause of behavioral changes limited to the US stimulus rather than the implant. Here, I will first cover some of the findings that discovered the possibility of sonogenetics. Then, I will cover some of the advances made in the field. Finally, I focus on the future potential use of sonogenetics.

4.3.1 TRP-4

Ibsen *et al.* [44] demonstrated that US can be used to modulate neurons in *C. elegans* expressing TRP-4. A knock-out of TRP-4 in specific neurons results in insensitivity to low-powered US. On the other hand, the addition of MBs, which amplifies the effect of US locally, increases the behavioral response to US stimulation. To study the behavioral response, the authors monitored two types of responses in the nematodes: *reversals* and *omega bends*. These responses were previously identified as related to the functionality of specific neurons of *C. elegans* [54]. Because *C. elegans* TRP-4 has no mammalian homologs, the authors [44] propose that introduction of the protein into mammals via genetic manipulation can introduce sensitivity to low-powered US. The MBs used in their experiments could be introduced via the blood, where they can remain without causing adverse effects to increase the effect of US. This approach's downsides include a relatively low lifespan of MBs in the blood stream. The authors proposed that other molecular targets might be better suited for this technique. Candidates include mechanosensitive channel of large conductance (MscL), a force sensor derived from *Escherichia coli* or other mutated channel-forming proteins [44].

4.3.2 MscL

Indeed, Ye *et al.* [55] provides evidence that a mutated version of MscL(I92L) can trigger action potentials in a rat's hippocampal cells after stimulation with low-pressure US. Stretching of the membrane causes a channel in wt MscL to open that allows ions and small molecules to pass while the mutation allows MscL(I92L) to be activated by US. The authors show that even without the presence of MBs, US can evoke action potentials in cultured cells. They showed this by examining voltage-clamped neurons exposed to US. Both neurons without MscL as neurons with wt MscL showed no current response, while neurons with I92L-MscL showed intensity-dependent response. Although low-pressure US is used in their experiments, it is not low-frequency US. High-frequency US has impaired penetrability and is therefore not directly applicable *in vivo*.

4.3.3 mPrestin

In an attempt to mitigate some of these limitations, Huang *et al.* [56] devised a tool that responds to low-frequency, low-pressure US using mouse prestin (mPrestin). mPrestin is a transmembrane protein thought to be responsible for US sensitivity in several echolocating bat species. The researchers found that introducing two point mutations (namely N7T and

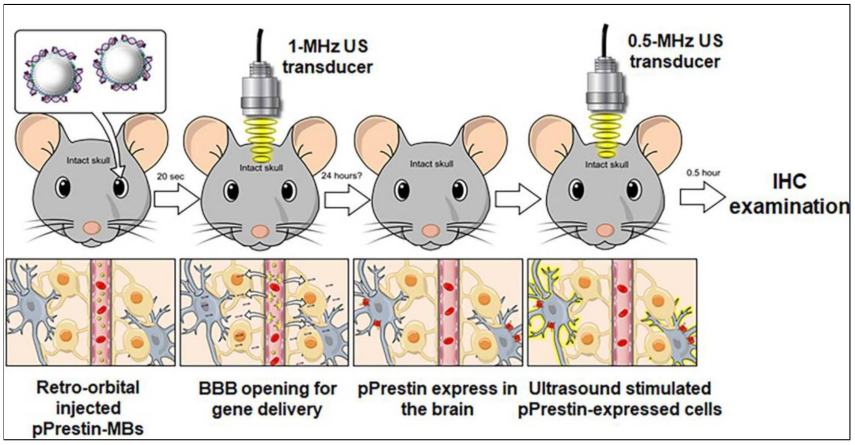

Figure 4.4: Experimental flowchart of US aided gene delivery [52].

N308S) further improved the sensitivity of mPrestin to low-pressure, low-frequency US. They also compared the sensitivity of mPrestin(N7T, N308S) to TRP-4 (as used by Ibsen *et al.* [44]) and found mPrestin(N7T, N308S) to be the most responsive to low-frequency US.

Other work from the same group aimed to improve the methods of gene delivery [57]. This is needed to make cells sensitive to US. Similar to approaches used in optogenetics, a common method to transfect specific neurons *in situ* is to use engineered adeno-associated viral particles [56]. [58] described the discovery that US can disrupt the blood-brain barrier (bbb) if amplified by MBs. If US is targeting the bbb, cavitation of MBs can cause the bbb to become permeable to plasmids. Wu *et al.* [57] used MBs loaded with plasmids containing mPrestin(N7T, N308S) to transfect mice neurons *in vivo*. The MBs serve two purposes in this experiment. First, they contain the payload, the plasmid, that will be introduced into the brain cells. Second, they contain a gaseous perfluoropropane core, which makes the MBs locally amplify the US stimulation. To start the experiment (illustrated in 4.4), the researchers inject the plasmid-loaded MBs into the blood. Then, a 1 MHz US transducer is used to target the area of interest. This triggers the MBs, which permeabilizes the bbb and bursts the MBs, which releases the plasmid into the brain. They then waited for mPrestin to be expressed in the brain, after which they used a lower frequency US transducer to stimulate mPrestin and were able to show that a Ca^{2+} influx had occurred in these cells. However, how exactly mPrestin stimulation with US results in a Ca^{2+} influx is as of yet unknown.

Chapter 5

Discussion on Literature

5.1 Electrical stimulation

ES is a promising area of research. Time and time again, researchers find improved methods and schemes for improved differentiation of stem cells. Similar to the field of study into NPs, the approaches are diverse. I pose this as a symptom of a field that is in rapid development. More efforts to improve this technique could help bring it closer to clinical applications.

As discussed, most, but not all, effects seem to be explained by changes in Ca^{2+} concentrations. Because of the ubiquity of Ca^{2+}-dependent pathways in cell types, this approach might pose challenges. Since electrical stimulation is not specific, and as such will affect all tissue surrounding the target cells. A variant of this approach that has not turned up in this literature study is the following: engineered targets that have been made sensitive to a specific type of otherwise harmless electrical stimulation. Such an approach would reduce the chances of inadvertent side effects if applied *in vivo*. A more relevant application of the technique is to direct ESCs to differentiate to NPCs and neurons *in vitro* and then introduce them to patients in a later stage. Although conceptualized, no one has executed this approach to my knowledge.

Electrically conductive hydrogels as substrate can help produce more homogenous results of differentiated cell types. This is because currents experienced by cells are more uniform compared to using less conductive materials.

5.2 Nanoparticles

The process by which NPs can enter cells is well understood. receptor mediated endocytosis (RME), phagocytosis, pinocytosis, and caveolar endocytosis are mechanisms that, depending on the size of the particle, can take up NPs.

However, as is evident from the varying mechanisms triggered by the particles once they enter a cell, the material coating the NP is hugely important for the downstream effects NPs have. Although ferritin, VA-CN and gold-coated NPs were all shown to increase differentiation in cells, the mechanisms the authors reported on varied considerably. It is likely that many other types of particles, too, affect differentiation without negatively impacting health.

As a relatively young field of inquiry in differentiation, investigations into the effect of NP are diverse. Approaches regarding fabrication, testing hypotheses, and models vary to such an extent that comparing their results is questionable. For the field to grow, scientists must make efforts to standardize the evaluation of these particles. Then, fair comparisons can be made between different types of particles based on their size, material, or treatment. Although the various differentiation markers used in the field provide their insight into the process, reporting a suite of markers, or even a standardized marker, would offer more insight. As it stands, NPs provides promising progress, but accurate comparisons are difficult.

5.3 Ultrasound

5.3.1 Discussion

I examined the current views on the mechanisms that underlie neuromodulation after stimulation with US. So far, researchers have found evidence for thermal and non-thermal effects, such as cavitation, changes in membrane capacitance, or stimulation of the Notch signaling pathway. Because the effects of US largely depend on the specific parameters chosen, US with different settings likely works via other mechanisms in various capacities.

Many efforts also examine the safety aspects of US stimulation. Many metrics are available to indicate US properties (in addition to physical properties of US), such as the various variants of intensity, MI and TI. MI and TI were developed in an attempt to relate US properties to tissue damage. Unfortunately, both are becoming increasingly less reliable in describing effects on tissue as the field progresses. The research described in this thesis has indicated that many effects manifest unrelated to temperature changes. MI was developed based on data from MBs experiments with short US pulses. The applicability of this measure on, for example, longer pulses has not been verified sufficiently [36]. Future work may be needed to develop measures that directly relate US properties to tissue damage.

I also looked at the advances in sonogenetics. In the five years sonogenetics has been explored, researchers have taken major strides to develop the technology further. After the initial experiments with TRP-4 [44], researchers have moved on to other proteins more sensitive to low-pressure US and experiments in larger animal models. MscL(192L) has been investigated and shows promising results *in vitro* [55], but has yet to be applied *in vivo*. Others have attempted to improve sonogenetics using mPrestin(N7T, N308S) [52, 56]. They also developed a tool to minimally invasively change gene expression *in vivo* using US.

A great deal remains to be done. Because one can see sonogenetics as a counterpart to optogenetics, comparing the two is essential. The development of sonogenetics is mainly fueled by the search for a non-invasive alternative to optogenetics. As discussed in **Section 4.1**, the invasive nature of optogenetics limits research applications and introduces animal harm. However, the temporal precision of optogenetics appears to be largely superior to sonogenetics. Where optogenetics can operate in less than a millisecond [59], thermal effects of ultrasound only operate on the seconds time scale, with non-thermal effects currently operating on a millisecond time scale [60].

5.3.2 Concluding

A new wave of research emerged after the proposal of sonogenetics as a research tool by Ibsen *et al.* [44]. Since then, considerable strides have been taken to improve the technique. The focus has shifted from providing *in vitro* proof-of-concept to *in vivo* applications. This transition required targets sensitive to low-frequency US because that type is barely attenuated by tissue, including bone. Ye *et al.* [55] and Wu *et al.* [57] performed research into such targets, while Huang *et al.* [56] proposed an alternate, non-invasive method of transfecting cells *in vivo* with high spatial accuracy. Lots of research is still ahead, too. The target found thus far are not as sophisticated as targets in optogenetics. For one, the proteins found thus far have limited *in vivo* validation in the case of MscL [55], or limited specificity in the case of mPrestin [57]. Other limitations include the temporal precision achievable by current sonogenetic approaches, which, compared to optogenetic approaches, is lacking (seconds for some approaches [60] versus less than a millisecond [59]). The search for targets sensitive to low-frequency, low-pressure US compounds that results in an influx of one specific ion in a temporal, exact manner remains active.

More research is needed to determine if this can be resolved, as the temporal precision of optogenetics is a fundamental reason for its widespread adoption. Part of the lagging state of sonogenetics compared to optogenetics can be explained by the difference in efforts put into both techniques. For example, the search term *optogenetics* yields over a thousand results on PubMed during 2019, while *sonogenetics* returns two results during the same time frame. Increasing the effort put into finding solutions for the problems sonogenetics faces compared to optogenetics may help make it the preferred option for researchers.

Part II

Practical Work

Chapter 6

Introduction on practical work

As laid out in **Chapter 4**, investigating the effects of US on differentiation is highly promising. However, certain gaps remain in our understanding of the interaction between US and NPCs. Part of the current gap in knowledge has to do with practical challenges.

Several materials are available that can generate US. Which type is best suited to generate the type of US required for NPC differentiation, however, is unclear. Further, NPC are commonly cultured in either glass or plastic wells. Can cells be subjected to US in the environment they are usually kept, or are adjustments needed? Similarly, would this type of experiment affect others kept in the same incubator?

To optimize the findings, equal exposure for most cells in a well is required. Unfortunately, the type of US transducer used best to expose cells as homogeneously as possible is not yet known.

6.1 Goal

6.1.1 Bigger picture

The bigger picture of this thesis is to gain insight into the effects of US of the differentiation of NPCs to neurons. Specifically, finding the optimal parameters to maximize neuron generation, starting from NSCs. Parameters to investigate could include exposure time, exposure intensity, and the number of cells to seed. Improving these parameters gives optimal chances for NPCs to differentiate into neurons.

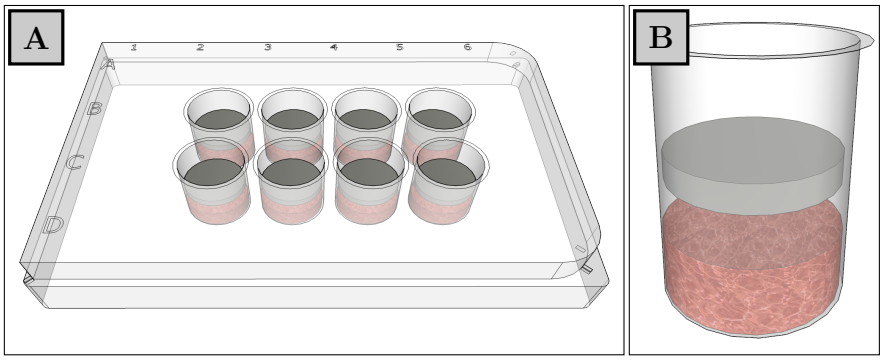

Figure 6.1: Experimental setup. A shows a modified 3D printed 24-well plate. Because microscopy analysis would not reach the outer wells, they are not populated. Wells contain NPCs on a gelatin substrate (red). Transducers (grey) are in contact with medium (not shown) and attached to the cover. **B** shows a close up of one well.

6.1.2 Feasibility

The experiments described here aim to explore the feasibility of investigating US to increase differentiation of NPCs to neurons. Two areas of interest were explored. Firstly, a section that encompasses making models to find parameters for transducer shape, transducer size, well size, material properties, and cell-substrate that allow for investigations mentioned in **Section 6.1.1**. These investigations are described in **Chapter 7**. Secondly, I validated findings from the literature that differentiating ESCs into NPCs is achievable with the available materials. This is described in **Chapter 8**.

Chapter 7

Modeling ultrasound transducer

The functionality of US transducers based on lead zirconate titanate [$Pb(Zr_xTi_{1-x})O_3$] (PZT), a piezoelectric material typically used for generating US waves, is strictly determined by its physical dimensions and shape and the voltage applied to the material. Therefore, exposing cells to the desired wavelength of US is vital. Therefore the optimal thickness and shape of the transducer were determined in advance using 3D finite element method (FEM) modeling software. Specifically, COMSOL Multiphysics with the Acoustics Module was employed to model the dimensions for the transducer with the medium for the cells, a backing material for the transducer, and the structure of the well. Per a previous study [57], the goal was to expose the cells to 1 MHz.

Because of the rotational symmetry of both the transducer and the well in which the cells will be placed, a 2D axisymmetric model was selected. Choosing this over a full 3D model allows for cheaper calculations while maintaining the same accuracy. In addition, a study was done in the frequency domain to determine the frequency of the PZT material resulting from electrical stimulation.

7.1 Near field of cylindrical beam

An unfocused beam of US will transmit a cylindrical-shaped beam near the transducer. Further away, however, the beam will converge into a focal point. The zone before the beam converges is referred to as the near-field, while the zone after is far-field. To allow equal exposure of all cells in the well to the same intensity of US, the goal is to construct a cylindrical US beam. Therefore, the cells must be exposed in the near field zone of the transducer. However, measuring the frequency response of the transducer is best done at the focal point. The theoretical focal point of an US transducer can be found via **Equation (7.1)** from Gao *et al.* [61].

$$F_n = \frac{r_T^2}{\lambda}, \text{ with } \lambda = \frac{c}{f} \tag{7.1}$$

where F_n is near field zone length, in m,
r_T is the radius of the transducer, in m,
λ is wavelength, in m,
f is frequency, in Hz,
c is wave velocity in the medium, in $\mathrm{m\,s^{-1}}$.

Applying this to my model, using the speed of sound in water at 20 °C, $c = 1480\,\mathrm{m\,s^{-1}}$, I get **Equation (7.2)**.

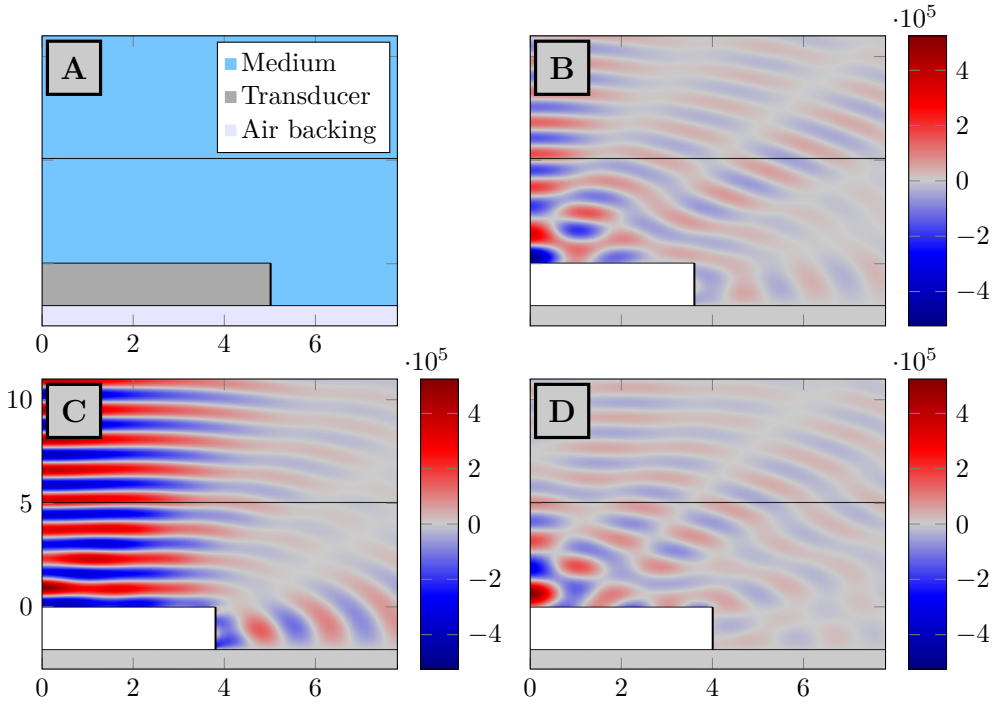

Figure 7.1: Results of COMSOL Multiphysics frequency simulations. A Visual representation of the geometry used for analysis in COMSOL Multiphysics, **B** Acoustic Pressure (AP) output for $t_d = 3.6\,\text{mm}$, **C** AP output for $t_d = 3.8\,\text{mm}$, **D** AP output for $t_d = 4.0\,\text{mm}$.

$$F_n = \frac{r_T^2 \cdot f}{1480\,\text{m}\,\text{s}^{-1}} \tag{7.2}$$

7.2 Simplified geometry

Revolving a rectangle around the z-axis results in a disc. Therefore, a rectangle starting in $(0, 0)$ is constructed with radius of the transducer (r_T) width and thickness of the transducer (t_T) height. Below the transducer, a backing material is placed with a thickness of 1 mm and 10 mm radius. On top of the transducer, a final rectangle is constructed up to 11 mm high and 10 mm wide (). Then, I set up a perfectly matched layer (PML), using COMSOL Multiphysics features of the Acoustics module. A PML can be described as a computation trick to simulate infinity. With it, the transition between two objects absorbs all simulated energy without reflecting anything. This approach allows me to analyze the waveform that results from the transducer in isolation without considering potential reflections from interactions. The medium's height allows for examining a large portion of the theoretically resulting waveform in line with the transducer. In **Figure 7.1A**, I illustrated the geometry. The medium's width was chosen to be 7 mm.

7.3 Frequency sweep

Commercially available piezoelectric material comes in a variety of thicknesses. Therefore, I simulated thicknesses $t_T \in \{2.03, 1.47, 1.01\}\,\text{mm}$ in the current setup and measured the corresponding acoustic intensity to the applied frequency in the theoretical focal point in COMSOL Multiphysics. Results are shown in **Figure 7.2**. Similar to the specifications supplied by the vendor, the material with $t_T = 2.03\,\text{mm}$ generates a wave with $f = 1\,\text{MHz}$,

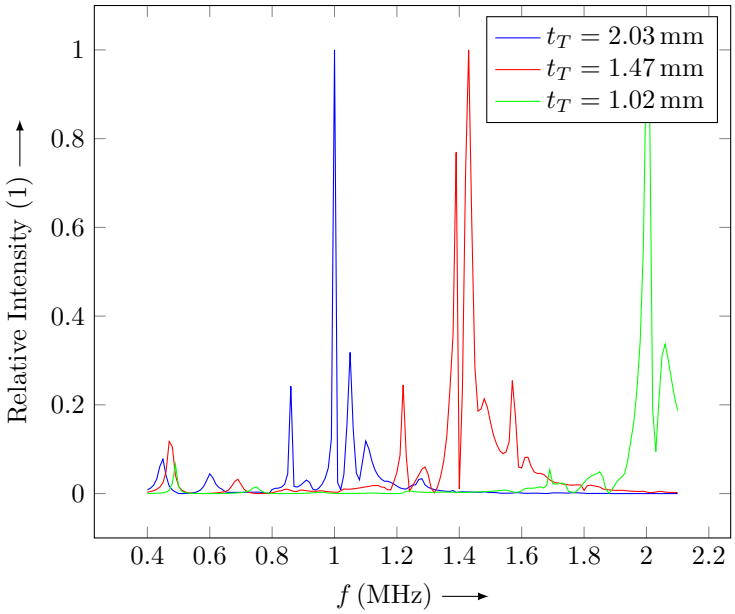

Figure 7.2: Frequency sweep measured in focal point.

and thus this thickness was selected for further simulations. The results from the simulation correspond to the reported frequency by the manufacturer.

7.4 Mesh refinement

For these simulations, unstructured quadrilateral meshes were generated. I chose to generate an unstructured mesh because the transducer and the medium have different requirements for precise modeling. Notably, the transducer needs high-density meshing to accurately model the piezoelectric effect, while the density requirement of the propagating sound waves is much lower. Because of the natural quadrilateral shape of the transducer, medium, and backing, I chose to generate a mesh with quadrilateral elements.

I performed a mesh refinement study to help determine the validity of the results from finite element analysis. The finer the mesh that can be used, the more accurate the results are with respect to the input. However, this comes with the caveat of extra computations. Therefore, I need to balance computational cost and acceptable accuracy. A mesh refinement study gives insight into this trade-off. If the results with a course mesh are similar to results with a finer mesh, I have found a mesh that is likely to produce accurate enough results with fewer computations. I, therefore, repeated the same study with an increasingly fine mesh. While increasing the mesh, the output I studied was the intensity at a 5 mm distance from the transducer. For this study I used $t_T = 2.03\,\text{mm}$ and $r_T = 3.8\,\text{mm}$. The meshing was performed by COMSOL, where I set the *maximum element size* parameter to λ/e with $e \in \{1, 2 \ldots 7\}$.

7.5 Radius optimization

The radius of a circular transducer can influence the response of a transducer. Specifically, the ratio between a transducer's radius and thickness determines its resulting waveform when excited [62]. In short, depending on the exact ratio between the dimensions, a vibrational mode is selected from a spectrum of vibrational modes. Describing these modes in detail warrants a full-size PhD thesis, for which I advise the reader to look into (Kocbach2000). The vibrational mode I am interested in is a piston-like movement. An optimally calibrated ratio between radius and thickness reduces side effects and poorly predictable behavior, including reflections.

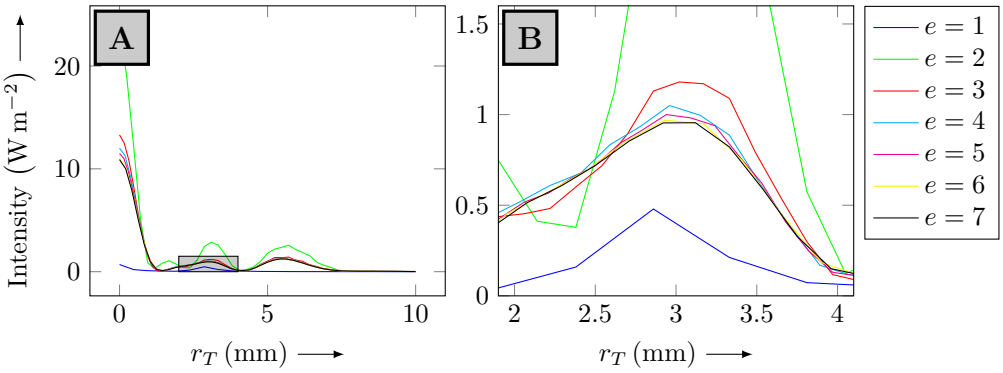

Figure 7.3: A mesh refinement experiment. e represents the number of elements per wavelength λ. **A** shows the full results with the rectangle representing the area that **B** shows of the same results. Starting from $e = 4$, the results converge and show no further increase in accuracy. $e = 5$ is chosen for future experiments.

Therefore, after having determined t_T and the required mesh fineness, I now look to optimize the radius of the transducer (r_T). Therefore, I simulated $t_T \in \{2.01, 2.02 \ldots 5.00\}$ mm and examined the generated *Acoustic Pressure* graphs. In figures **Figure 7.1B**, **Figure 7.1C** and **Figure 7.1D** I show the results of simulation with $t_d = 3.6$ mm, $t_d = 3.8$ mm, $t_d = 4.0$ mm, respectively. Where $t_d = 3.6$ mm and $t_d = 4.0$ mm show chaotic patterns with unpredictable spots with high intensity, $t_d = 3.8$ mm shows a standing wave. Standing waves maximize the number of cells exposed to the same stimulation. Further, they increase reproducibility by removing chaotic patterns emerging from unpredictable reflections of the plastic material. Therefore, the results shown in **Figure 7.1C** best suit this experiment, and I take $t_d = 3.8$ mm as my result for further analysis.

7.6 Complex geometry

A more complex model will help account for plastic material properties, the gelatin layer, and the surrounding air. This approach helps anticipate potential issues concerning sound reflection back into the well and prevents this experiment from influencing others in an incubator.

In addition to the simple model, the complex model has the following features: a gelatin layer, a well, and a layer of air. The layer of gelatin replaces part of what used to be simulated by medium only. The top-most part, 1 mm in height, is now simulated with gelatin material properties. The well is constructed with a 2 mm thick rectangle beside the medium and directly above the gelatin layer. Finally, 1 mm of air is simulated surrounding the entire object, with associated PML properties.

Again, I simulated $t_T \in \{2.01, 2.02 \ldots 5.00\}$ mm and examined the generated *Acoustic Pressure* graphs. I compared two material properties for the well: plastic (**Figure 7.4B**) and polydimethylsiloxane (PDMS) (**Figure 7.4C**). The results from the simulation with the plastic material show a chaotic pattern, whereas the results from the PDMS simulation show a standing wave. With everything else equal, I conclude that the material properties of the well are vital to allow for the construction of standing waves.

7.7 Custom 24-well plate

The COMSOL Multiphysics experiments described above showed that the plastic material used in the wells was too reflective for use with US with $f = 1$ Hz. Therefore, I created a 3D-printable mold. The design is available from `www.github.com/djangobrunink/thesis`.

The choice of material to fill the mold with is PDMS, a non-reactive, organic polymer transparent to US. Further, this material is cheap, easy to produce, and easily moldable [63].

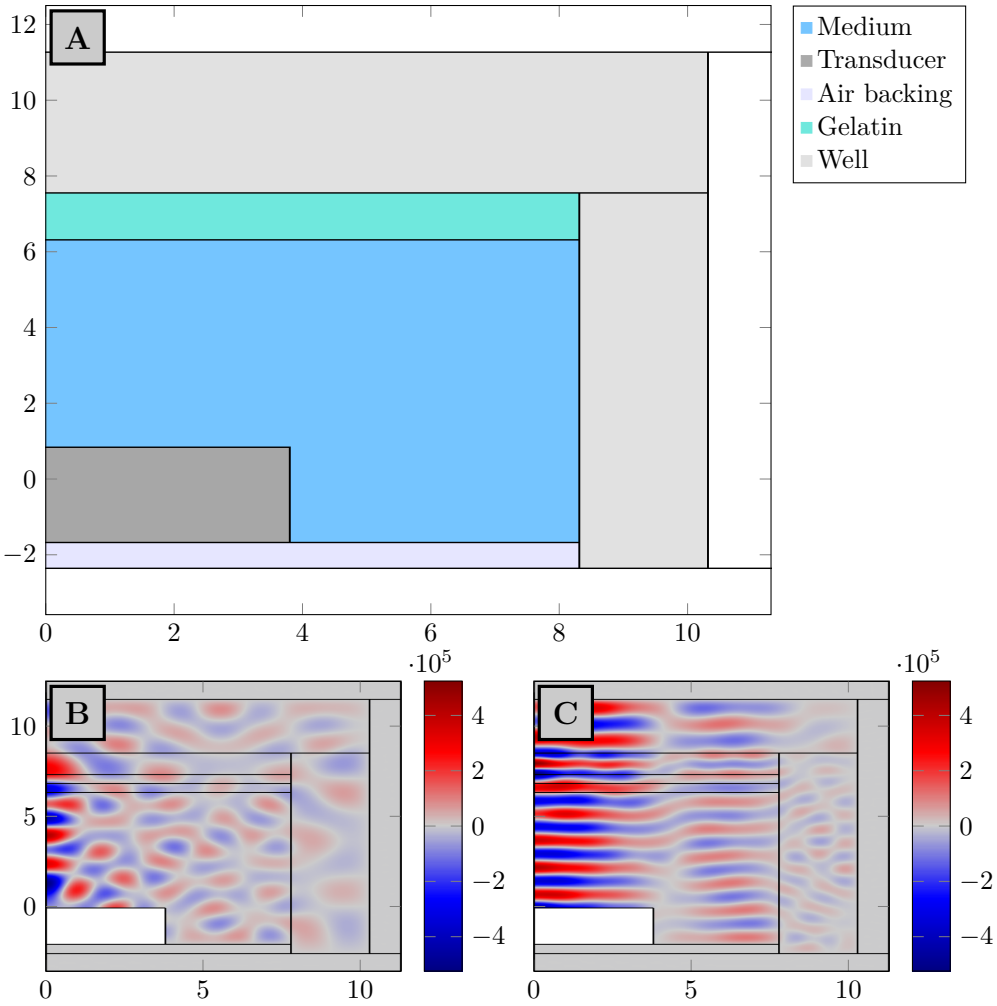

Figure 7.4: Detailed model of a transducer in a well.

However, long-term cell adhesion, proliferation, and differentiation are challenging due to the hydrophobic nature of PDMS. As such, I propose to use a solution provided by Chuah *et al.* [63]: coating PDMS with a dopamine solution with pH = 8.5. Cells grown on the resulting poly-dopamine (PD) structure were 40-fold more adherent to the substrate. Usage of PD-coated-PDMS is, therefore, a promising candidate as a substrate for NSCs.

Figure 7.5: Intended usage of 3D printable mold.

Chapter 8

Cell culture

8.1 Method

8.1.1 Cells

The cells used in this project are mouse ESCs, specifically of the E14TG2a cell line provided by Hyun Youk (TU Delft). The protocol is adapted from Gaspard *et al.* [6] and Gazina *et al.* [7].

8.1.2 Growth medium

Cells were grown with DMEM + glucose (Gibco), enriched with 15 % fetal bovine serum (FBS) (Gibco), 1 mM sodium pyruvate (Gibco), 0.1 mM β-mercaptoethanol (Sigma-Aldrich), 1x non-essential amino acids (Gibco), 1x GlutaMAX (Thermo Fisher), 1000 U mL^{-1} mouse recombinant leukemia inhibitor factor (Stem Cell Technologies) and 50 U mL^{-1} of penicillin/streptomycin (Gibco). It will be referred to as *ES medium*.

8.1.3 Differentiation medium

To trigger cells to start differentiating to NPCs, cells were cultured in DMEM/F12 + GlutaMAX (Gibco) enriched with 1 % N2 supplement (Gibco), 1 mM sodium pyruvate (Gibco), 1x non-essential amino acids (Gibco), 1x GlutaMAX (Thermo Fisher), 0.1 mM β-mercaptoethanol (Sigma-Aldrich), 500 µg mL^{-1} bovine serum albumine, fraction V (Gibco) and 50 U mL^{-1} of penicillin/streptomycin (Gibco). The medium was filtered before use and will be referred to as *defined differentiation medium (DDM)*.

8.2 Growth

ESCs were incubated in ES Medium for six to twelve days and passed thrice weekly, as illustrated in **Figure 8.1**. The six-day minimum is needed to allow the cells to relieve stress factors, while the twelve-day maximum ensures their passage number does not increase too

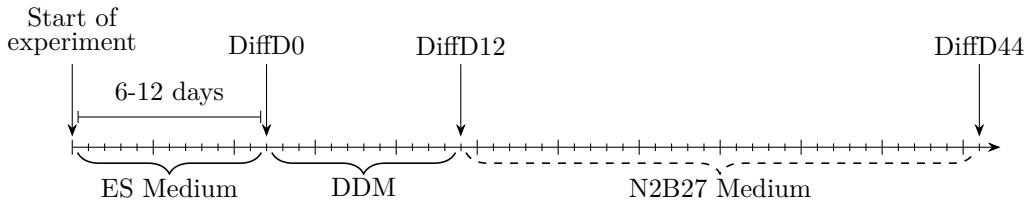

Figure 8.1: Schematic representation of the planned conditions cells were exposed to.

much. Then, at differentiation day 0 (DiffD0), the medium of cells was changed to DDM. Every 48 h, medium was refreshed. Although not performed here, when differentiating into neurons, at DiffD12 medium DDM would be replaced by N2B27 medium.

Chapter 9

Discussion

9.1 Future work

This work lays a foundation that can be used to further explore the effects of US onto stem cells (SCs). However, further work is required to provide a definitive answer concerning the viability of US exposure to improve neuron yield in lab conditions.

Two steps must be taken before the desired experiments can be carried out.

Firstly, in this work, I investigated the parameters that an US transducer must have to be used for experimentation. Now, the specified parts must be constructed and assembled.

Secondly, the 3D printed mold should be used to fabricate a well that allows for testing samples with US without the adverse effects of reflections of the waves interacting. Then, some practical issues must be verified. For example, the new well must withstand the temperature in an incubation chamber and the effect of ultraviolet irradiation for sterilization purposes. Following that, it must be verified that cells can grow and differentiate in this new well.

Finally, with these prerequisites in place, the main experiment can take place. That is, the parameters for optimal neuron growth in lab conditions can be determined. Primarily, parameters of US stimulation as TT, BD, burst interval (BI), PL and 1 / pulse repetition frequency (1/PRF) can be optimized (**Figure 4.1** is available for reference). Optimized values for all these parameters maximize neuron growth. In **Figure 6.1**, I illustrated how an experimental setup could look.

9.2 Conclusion

Neuronal cells are difficult to culture in lab conditions from SCs. Numerous approaches have been attempted to improve the yield of neurons. One such approach is that the development of neuronal cells during the natural development of the brain is influenced by US. Applying US onto NSCs during development could improve the yield of NSCs developing into neurons. In this thesis, I set out to provide a basis on which further research can be done.

Achieving better neuron yields in lab conditions decreases the number of animal models required to study neurons. Of course, this is a goal in itself. However, since animal studies often require much time and effort to set up, it could also reduce the time needed to investigate hypotheses concerning the development of neurons. Further, more facilities can process cell cultures compared to animal models, increasing the accessibility to such studies and decreasing research time.

In order to expose developing SCs to US, I cultured murine ESCs into NPCs. Then, an US setup containing PZT could expose the cells to ultrasound during development. Simulations were performed in COMSOL Multiphysics to examine the effect of US onto plastic wells.

The nature of plastic wells is such that US waves in the range expected to affect the cells optimally are reflected into the sample, disrupting organized wave patterns that allow for constant, even exposure.

A 3D-printed mold was developed to create custom 24-well plates made of a material that does not interfere with US waves. A good candidate for material choice is PD-coated-PDMS, as discussed in **Section 7.7**.

Simulations of sound waves can provide good insight into actual results. However, the simulations performed for this study assume the materials to be perfectly uniform and of the precise dimensions entered into the equations. Slight changes to the input can cause relatively large changes to the output. During experimentation on cells with US, minor modifications likely will have to be made.

With the work described in the thesis, the next step is to expose cells in the custom 24-well plate to US generated by the PZT.

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