Do Nanoparticles Affect Neuronal Function?
- Establishment of a Microelectrode-Based Assay

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Abstract

Nanomaterials are used extensively in industry and daily life but comparatively little is known about the possible health effects. Silver nanoparticles (AgNPs) and gold nanoparticles (AuNPs) are two metal nanoparticles that have been intensively studied. AgNPs due to their antibacterial effect and AuNPs due to good intrinsic properties and the fact that they are highly biocompatible. Extracellular recordings of spontaneous electrical activity from *in vitro* cultured neurons using microelectrode arrays (MEAs) can be used in order to study if AgNPs and AuNPs affect neuronal function. Neuronal cell cultures were exposed to AgNPs and AuNPs with a diameter of either 20 nm or 80 nm in low concentrations (800 particles/cell). To this date, neurotoxicity assessment still relies on *in vivo* animal testing. However, human induced pluripotent stem cell (hiPSC)-derived neurons (iCell® Neurons commercially) were used in this thesis since animal experiments have several drawbacks and do not always mimic the human physiology. Immunocytochemical analysis of the iCell® Neurons as well as images taken with a scanning electron microscope concludes that the cells develop into functional neuronal networks and attach to electrodes on the MEAs. This provides a good basis for electrophysiological recordings. Significant multiunit extracellular activity associated with spontaneous action potential waveforms was recorded from the iCell® Neurons with the setup presented in this thesis. However, no quantitative analysis of how AgNPs and AuNPs affect the neuronal function was carried out due to a lack of experimental data. Only 10% of the electrodes on the MEAs recorded neuronal activity and further studies are needed in order to develop a fully functioning assay. Nevertheless, hiPSC-derived neurons cultured on MEAs may still enable animal free neurotoxicity testing in the future and could be a valuable tool when studying possible health effects of nanoparticles and other compounds.

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List of Abbreviations

AgNP	silver nanoparticle
AP	action potential
AuNP	gold nanoparticle
CMOS	complementary metal oxide semiconductor
CNS	central nervous system
DCX	doublecortin
DIV	day/days *in vitro*
EAP	extracellular action potential
ECM	extracellular matrix
GABA	γ-aminobutyric acid
GFAP	glial fibrillary acidic protein
hbFGF	human basic fibroblast growth factor
hEGF	human epidermal growth factor
hiPSC	human induced pluripotent stem cell
hLIF	human leukemia inhibitory factor
hNPC	human neural progenitor cell
ICC	immunocytochemistry
ITO	indium tin oxide
KSR	KnockOut Serum Replacement
LFP	local field potential
MAP2B	microtubule-associated protein 2B
MEA	microelectrode array
NBA	Neurobasal-A
NP	nanoparticle
PBS	phosphate buffered saline
PCA	principal components analysis
PCL	poly-ε-caprolactone
PEI	polyethyleneimine
PFA	paraformaldehyde
PLL	poly-L-lysine
PSD95	post-synaptic density 95
RT	room temperature
SEM	scanning electron microscopy
SiN	silicon nitride
SNR	signal-to-noise ratio
TiN	titanium nitride
vGluT-2	vesicular glutamate transporter 2

Do Nanoparticles Affect Neuronal Function? - Establishment of a Microelectrode-Based Assay

Popular Scientific Summary

Despite the advances in nanotechnologies and the abundance of nanoparticles in commercial applications comparatively little is known about possible health effects. If nanoparticles of gold and silver affect the function of neurons is still a question that remains to be answered.

Nanoparticles are objects that are in the range of 1-100 nm in at least one dimension. Nanoparticles are so small that they can enter the human body through a variety of different ways and they are also able to cross the blood-brain barrier and reach the central nervous system (CNS). This could introduce new unwanted toxic effects. Gold and silver nanoparticles are two very common and intensively studied nanoparticles. Silver nanoparticles, due to their antibacterial effect, are used in *e.g.* cosmetics, textiles and household goods whereas gold nanoparticles are used to create new drug and gene delivery applications.

The CNS consists of the brain and spinal cord where neurons and glial cells are the basic cell types. Glial cells provide support and protection for neurons. The neurons in the central nervous system, however, control all major functions of the human body by processing and transmitting information with a specific type of electric signal, an action potential.

Action potential waveforms can be recorded and studied by culturing neuronal cells directly on top of microelectrodes. When electrical activity occurs, ions flow across the cell membrane. The moving ions generate changes in the electric field which in turn can be recorded by the metal microelectrodes. Arranging several microelectrodes in an array creates a microelectrode array (MEA). MEAs enable simultaneous recording of extracellular activity from a large number of neurons. The MEAs used in this thesis to record neuronal activity before and after exposure to nanoparticles have 60 microelectrodes with a diameter of 10 µm.

To this date, neurotoxicity assessment still relies on animal experiments. However, human cells were used in this thesis since animal tests are expensive, time consuming and ethically debatable. Human neural progenitor cells (hNPCs) was the first cell type that was tested. hNPCs are neural stem cells taken from a human embryo and these cells can differentiate into cell types that populate the CNS. No significant neuronal activity was recorded from the hNPCs with the MEA plates. Therefore, human induced pluripotent stem cell (hiPSC)-derived neurons (iCell® Neurons commercially) were used instead. hiPSCs are generated from adult human cells. The cells have been reprogrammed into stem cells that in turn can develop into any cell type in the human body, in this case neurons.

Significant electrical activity was successfully recorded from the iCell® Neurons. Action potential waveforms from several different neurons were able to be recorded simultaneously with the MEAs. For instance, one microelectrode recorded activity from four different neurons at the same time. However, only 10% of the microelectrodes on the MEAs showed signs of neuronal activity. This means that no quantitative analysis of how gold and silver nanoparticles affect neuronal function could be carried out. Further studies are needed in order to improve the experimental setup. In conclusion, MEA recordings of hiPSC-derived neurons do show a lot of promise and may enable a new approach to efficient animal free neurotoxicity testing in the future and might very well turn out to be a valuable tool when investigating nanoparticles and other compounds.

Introduction

Electrophysiological recordings of *in vitro* cultured neurons exposed to gold and silver nanoparticles is the main focus of this thesis. This thesis also includes immunocytochemical studies of neuronal cultures as well as imaging with scanning electron microscopy. Therefore, this thesis begins with an overall introduction to neuronal cells and the generation of action potentials as well as the basics of electrophysiological measurements and neurotoxicity testing. Relevant nanoparticles, cell types and cell tools used in the experimental part of the study are also introduced.

Generation of Action Potentials

The central nervous system (CNS) consists of the brain and spinal cord where neurons and glial cells are the basic cell types. Neurons in the CNS control all major functions of the human body by processing and transmitting information with electric signals. Glial cells have important functions such as providing support and protection for neurons. Astrocytes are one of the most abundant subtypes of glial cells in the CNS and they outnumber neurons by over fivefold (Sofroniew and Vinters, 2010).

All different types of neurons contain four distinct regions with differing functions; the cell body, the dendrites, the axon and the axon terminals (smaller branches of the axon that form the synapses) (Figure 1). The axon is specialized for the conduction of a specific type of electric signal, an action potential, from the cell body, originating from the axon hillock, toward the axon terminal (Lodish et al., 2000). Chemical synapses transmit signals by the release of neurotransmitters from the axon terminal to *e.g.* a dendrite of another neuron.

An action potential is a series of sudden changes in the electric potential across the plasma membrane (Figure 1). Opening and closing of voltage gated cation channels in the plasma membrane results in a large ion flux over the membrane leading to an action potential. The membrane potential can be as much as +50 mV (inside positive) at the peak of an action potential (Lodish et al., 2000). Following the depolarization of the membrane that lead to an action potential a rapid repolarization occurs which in turn brings back the membrane potential to its resting state. The electric potential is approximately -60 mV (the inside negative relative to the outside) across the axonal membrane in the resting state (Lodish et al., 2000).

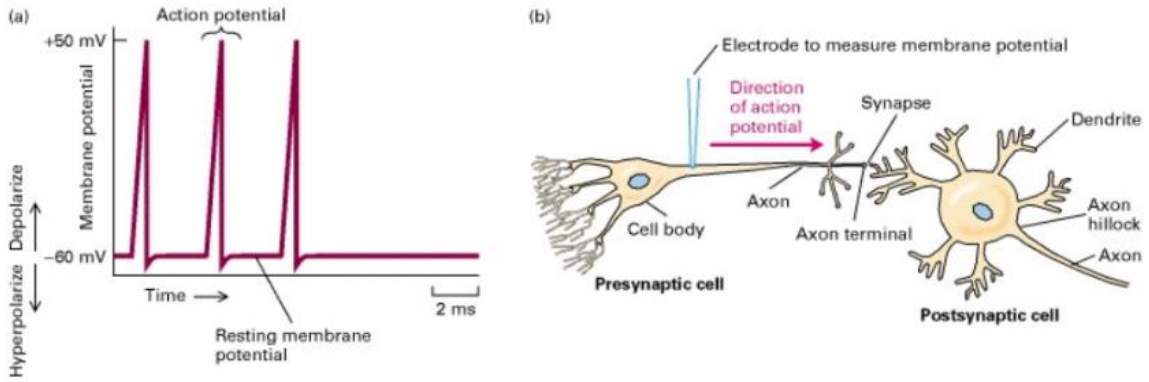

Figure 1 (Lodish et al., 2000). *(a) Recording of the axonal membrane potential during generation of action potentials. (b) The membrane potential of a presynaptic neuron is measured by inserting a small electrode into the plasma membrane.*

Electrophysiological Measurements

As mentioned previously, voltage gated ion channels are responsible for the generation of action potentials. Ion channels open and close in a stochastic fashion which can result in spontaneous action potentials, even in the absence of stimulation (O'Donnell and van Rossum, 2015). Spontaneously active neural networks in culture have been proposed as an efficient and sensitive model system to study neurotoxicity of chemicals (Gramowski et al., 2010). This is a simplified model of the CNS useful for electrophysiological studies of neurons. The measurements of spontaneous activity in neurons can be either intracellular or extracellular.

Intracellular measurements can be conducted with the so-called "patch clamp" technique, *i.e.* accessing the cell interior or cytosol with an electrode (Hierlemann et al., 2011). The patch clamp technique is an invasive method (puncturing the cell membrane), limited in cell viability time and in the number of cells that can be investigated simultaneously. The technique is therefore often limited to only looking at a few neurons per experiment (Obien et al., 2015). However, the patch clamp technique yields very accurate information about the electrophysiological properties of entire neurons (Hierlemann et al., 2011).

Extracellular measurements can be conducted with external microtransducers (Hierlemann et al., 2011). The neurons are cultured directly onto the electrodes or sensors. When electrical activity occurs, ions flow across the cell membrane. The moving ions generate changes in the electric field that can be recorded by metal microelectrodes. Extracellular recordings are non-invasive and can be used for long-term measurements (Hierlemann et al., 2011). Arranging many electrodes in an array creates a microelectrode array (MEA). MEAs allow simultaneous recording of extracellular activity from a large number of neurons. The simultaneous gathering of action potential and field potential data during long periods of time makes MEAs well suited for neurotoxicity screening (Johnstone et al., 2010). These features also allow scaling to high-throughput toxicity screening systems. See Figure 2 for a comparison between the appearance of recorded extracellular action potentials (EAPs) and intracellular action potentials (APs). The EAPs in Figure 2 are extracted from raw recordings of electrical activity containing local field potentials (LFPs) as well.

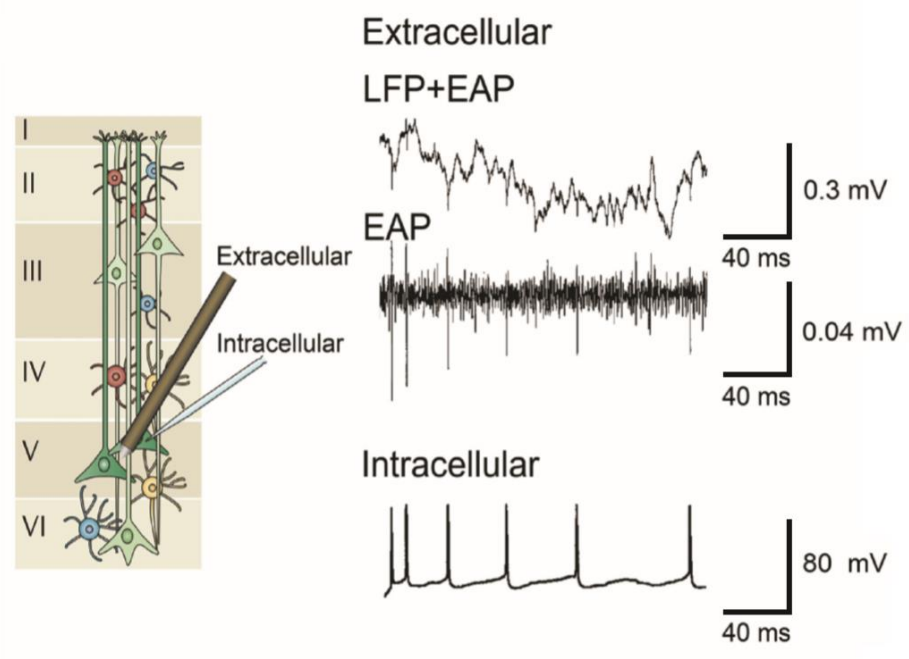

Figure 2 (Obien et al., 2015). *Modified image showing recordings of electrical activity with a tetrode (extracellular) and a glass micropipette (intracellular). The EAPs extracted from the raw tetrode recordings (LFP+EAP) correlate with the intracellular APs from a pyramidal cell. Note the differences in amplitude on the scale bars from the EAPs (0.04 mV) and the intracellular APs (80 mV).*

Typical MEAs for *in vitro* use consist of a glass slide or "chip" with an integrated array of extracellular microelectrodes, each insulated from each other and coated with conductive materials (Johnstone et al., 2010). These conventional MEAs rely on individual wiring of each electrode to off-chip amplifiers. This limits the electrode density and the number of electrodes on each MEA plate. The number of electrodes is typically between 64 and 512 on conventional MEAs (Maccione et al., 2015). MEAs that do not rely on individual wiring have been developed using CMOS (complementary metal oxide semiconductor) technology and post-processing micro- or nanotechnologies. CMOS-based MEAs can have more than 4000 closely spaced electrodes (Maccione et al., 2015). Conventional MEAs are now regarded as low-density MEAs due to the rapid development of new MEA plates. Approximately 15 years ago 60 recording electrodes was considered as many (Stett et al., 2003). However, recording activity from thousands of neurons simultaneously creates enormous data sets which become challenging to analyze (Maccione et al., 2015).

Data Analysis

When analyzing recorded extracellular signals from neuronal cultures, the signals from different neurons need to be identified and separated from the noisy voltage signals recorded by the electrode. This task is known as spike sorting and it is a necessary step for further data analysis. There is no generally agreed-upon best method for sorting spikes even though MEA recordings are becoming more commonly used (Swindale et al., 2017). Neurons being located very close together can make spike sorting more difficult since the recorded signals are likely to be very similar and hard to distinguish. Another factor which can cause problems is that the recorded signal from a neuron can vary over time due to *e.g.* migration of cultured neurons. Using MEAs with closely spaced electrodes (20-100 µm) can solve some of the problems since the spatial definition is improved (Swindale et al., 2017).

However, there is still a need for a spike sorting method that is quick, reliable and easy to use when converting raw data to sorted spike trains (Swindale et al., 2017). This is particularly important when analyzing large data sets with electrical activity from a large number of neurons recorded by a multitude of electrodes.

Neurotoxicity Testing

Neurotoxicity can be defined as any adverse effect on the structure, function or chemistry of the nervous system at maturity or during development that is induced by chemical or physical influences (Costa, 1998). Neurotoxicity are important adverse health effects of pharmaceutical drugs, environmental toxins, occupational chemicals and natural toxins (Costa et al., 2011).

To this date, neurotoxicity assessment still relies on *in vivo* animal testing (Tukker et al., 2018). Guidelines for *in vivo* testing have been developed, implemented and validated (Costa et al., 2011). However, animal experiments are expensive, time consuming and ethically debatable. Furthermore, animal-based models do not always mimic the human physiology and *in vivo* experiments are not well suited for high-throughput toxicity screening (Tukker et al., 2018). There is therefore a need for efficient *in vitro* strategies in neurotoxicity screening, preferably using human cells to avoid interspecies translation. Cells from rats or mice are commonly used for *in vitro* cell culturing. This thesis will, however, focus on two different human cell alternatives; human neural progenitor cells (hNPCs) and human induced pluripotent stem cell (hiPSC)-derived neurons.

Human Neural Progenitor Cells

Human neural progenitor cells (hNPCs) are neural stem cells, in other words, the progenitor cells of the central nervous system (CNS). The neural progenitor cells can differentiate into cell types that populate the CNS. The hNPCs used in this thesis are taken from forebrain tissue of a human embryo and have been established as a mitogen expandable cell line. When differentiated, these hNPCs form the three main phenotypes of the CNS, which are neurons, astrocytes and oligodendrocytes (Carpenter et al., 1999).

Human Induced Pluripotent Stem Cells

Human induced pluripotent stem cells (hiPSCs) are generated from adult human somatic cells, *e.g.* dermal fibroblasts (Takahashi et al., 2007). hiPSCs can be differentiated into neurons or any other cell type in the human body. hiPSC-derived neurons are commercially obtainable and available in high quantity but have not been used extensively in neurotoxicity screening and are not that well characterized for electrophysiological studies (Tukker et al., 2018). hiPSC-derived neurons can form functioning neuronal networks containing inhibitory and/or excitatory neurons without supportive cells such as astrocytes and oligodendrocytes. In comparison with hNPCs, the hiPSC-derived neurons are more expensive whereas the process of forming a functioning neuronal network is much faster. The hiPSC-derived neurons are a backup if electrophysiological recordings with the hNPCs are deemed inadequate.

Nanoparticle Toxicity

Nanotechnology is a major research area and engineered nanomaterials are used extensively in industry and daily life. Nanomaterials, including *e.g.* nanotubes, nanofibers and nanoparticles (NPs), are objects that are in the range of 1-100 nm in at least one dimension (Gramowski et al., 2010; Soderstjerna et al., 2013; Thorley and Tetley, 2013) and show strikingly different properties compared to the bulk material (*e.g.* increased surface area to volume ratio) (Cupaioli et al., 2014). In the field of nanobiomedicine, NPs can *e.g.* be used in order to create a safer and more efficient drug delivery approach (Soderstjerna et al., 2013; Thorley and Tetley, 2013) and in daily life NPs can be found almost everywhere (*e.g.* in cosmetics, paints, clothing and electronics) (Gramowski et al., 2010; Soderstjerna et al., 2013; Cupaioli et al., 2014). Despite the advances in nanotechnologies and the abundance of NPs in commercial applications comparatively little is known about possible health effects. NPs can, due to their small size, enter the human body through skin, airways, digestive tract and blood and they can also cross the blood-brain barrier to reach the CNS (Cupaioli et al., 2014). This could introduce new unwanted toxic effects since the larger surface area to volume ratio of NPs makes them more reactive (Soderstjerna et al., 2013).

Silver nanoparticles (AgNPs) and gold nanoparticles (AuNPs) are two metal NPs that have been intensively studied. AgNPs, due to their antibacterial effect, are used in coatings of medical devices and prothesis as well as in cosmetics, textiles and household goods (Soderstjerna et al., 2014). Approximately 30% of all NP-enabled products contain AgNPs (Haase et al., 2012). AuNPs have good intrinsic properties such as high chemical stability, surface functionalization and well-controlled size. AuNPs are also highly biocompatible, making them useful in drug and gene delivery applications (Soderstjerna et al., 2013).

Studies with an hNPC-based assay conclude that both 20 and 80 nm Au- and AgNPs can be taken up into the intracellular space when exposed to the cells in very low concentrations. AgNPs cause a significant stress response in growing hNPCs by affecting cell proliferation and apoptotic cell death. AuNPs did not significantly affect cell proliferation or apoptotic cell death. Morphological changes in hNPCs due to exposure to AuNPs were however observed (Soderstjerna et al., 2013). Previous studies with hNPC-based assays did not include electrophysiological recordings of neurons exposed to Au- and AgNPs. In another study, done on primary mixed neural cell cultures, AgNPs have been shown to affect functional processes in neuronal networks. AuNPs were used as control and did not affect the neuronal networks (Haase et al., 2012).

In electrophysiological studies on co-cultures of primary neurons and glial cells it has been shown that NP exposure induce acute functional neurotoxicity at low particle concentrations (0.03-100 $\mu g/cm^2$). The NPs studied in this case were carbon black, hematite (Fe_2O_3) and titanium dioxide (TiO_2). This study was done by using MEA neurochips (5 x 5 cm^2 glass chips, central 1 mm^2 recording matrix with 64 gold electrodes) (Gramowski et al., 2010).

Immunocytochemistry

Immunocytochemistry (ICC) is a very valuable tool in biomedical research used to identify proteins and other macromolecules in tissues and cells (Burry, 2011). ICC can be used in order to characterize neuronal networks and for assessing changes in morphology and the rate of differentiation of neurons when exposed to possibly neurotoxic compounds. Primary antibodies are used in order to target an antigen or a peptide associated to a specific protein in the neuronal culture. This enables the possibility of determining if a specific protein is being expressed by the cell culture or not. Proteins expressed by *e.g.* progenitor cells (Messam et al., 2000), immature or early neurons (Englund et al., 2002; Piens et al., 2010), neuronal synapses (Harper et al., 2017), inhibitory or excitatory neurons (Brady and Jacob, 2015; Yu et al., 2018) and glial cells (van Bodegraven et al., 2019) can be targeted in order to gain information about the cultured neuronal networks. Secondary antibodies with fluorophores, that bind to the primary antibodies, are used to visualize the targeted structures in a fluorescence microscope.

Scanning Electron Microscopy

Scanning electron microscopy (SEM) is a high-resolution imaging technique in which an electron beam is used instead of a light source in order to visualize structures on the micro- and nanoscale. Light microscopy is of great importance to scientific research, however, optical microscopes have a resolution limit of approximately 200 nm (Zhou et al., 2006) which is insufficient when studying nanomaterials.

SEM can be used for morphological studies of *in vitro* cultured cells (Boyde et al., 1972; Gramowski et al., 2010; Soderstjerna et al., 2013). Due to the nanometer resolution of modern SEMs, visualization of metallic nanoparticles is possible without almost any additional preparation step (Goldstein et al., 2014). However, imaging of biological materials requires a complicated preparation procedure including *e.g.* fixation, dehydration, drying and metal coating of the biological samples. Coating biological samples with a conductive material is needed in order to decrease charging artefacts and radiation damage in the course of SEM imaging (Goldstein et al., 2014). However, the metal coating materials available are usually composed of nanometer sized clusters. This can cause problems when imaging nanoparticles in cell cultures since the nanoparticles can be difficult to distinguish from the coating material.

Goal

In this thesis the goal was to develop an assay to evaluate the effect of gold and silver nanoparticles on neuronal function using *in vitro* brain models.

Aim

The specific aims of the study were to record changes in electrical activity of *in vitro* cultured human neural progenitor cells and human induced pluripotent stem cell-derived neurons caused by exposure to gold and silver nanoparticles using microelectrode arrays. The study also aims to investigate changes in morphology and differentiation potential of human neural progenitor cells caused by exposure to gold and silver nanoparticles using immunocytochemistry and scanning electron microscopy. The same techniques will also be used in order to study the human induced pluripotent stem cell-derived neurons.

Research Questions

1. Can an *in vitro* assay for assessing electrophysiological function of hNPCs be developed using MEA plates?

2. Is the electrophysiological function of hNPCs affected by exposure to AuNPs and AgNPs in low concentrations?

3. Is the morphology and differentiation of hNPCs affected by exposure to AuNPs and AgNPs in low concentrations? ICC and SEM will be used for this evaluation.

4. Can an *in vitro* assay for assessing electrophysiological function of hiPSC-derived neurons be developed using MEA plates?

5. Is the electrophysiological function of hiPSC-derived neurons affected by exposure to AuNPs and AgNPs in low concentrations?

6. What are the phenotypic characteristics of hiPSC-derived neurons and does exposure to AuNPs and AgNPs in low concentrations affect the morphology? ICC and SEM will be used for this evaluation.

7. How should the large data files from electrophysiological MEA measurements be analyzed?

Materials and Methods

Chemicals

L-glutamine, glucose, heparin, human leukemia inhibitory factor (hLIF), poly-L-lysine (PLL) solution, Terg-A-Zyme solution, laminin, 50% polyethyleneimine (PEI) solution, sodium tetraborate and bovine serum albumin were obtained from Sigma-Aldrich (St Louis, MO, USA). DMEM/F12 medium, fetal bovine serum and boric acid was obtained from Thermo Fisher Scientific (Waltham, MA, USA). iCell® Neurons Maintenance Medium and iCell® Neurons Medium Supplement were obtained from Cellular Dynamics International (Madison, WI, USA). N2-supplement, human basic fibroblast growth factor (hbFGF) and penicillin-streptomycin was obtained from Invitrogen Ltd (Paisley, UK). Human epidermal growth factor (hEGF) was obtained from ProSpec (Rehovot, Israel). Neurobasal-A (NBA) medium and KnockOut Serum Replacement (KSR) were obtained from Life Technologies (Carlsbad, CA, USA). Vectashield mounting medium with 4′,6-diamidino-2-phenylindole (DAPI) was obtained from Vector Laboratories, Inc. (Burlingame, CA, USA).

Cell Cultures

Expansion, Dissociation and Differentiation of Human Neural Progenitor Cells

The human neural progenitor cell line (hNPC) used in this experiment was originally established by L. Wahlberg, Å. Seiger and colleagues at the Karolinska University Hospital, Stockholm, Sweden (original work with the hNPCs is described in (Carpenter et al., 1999)). This cell line was kindly provided by Prof. A. Björklund (Dept. Exp. Med. Sci., Lund University, Sweden). hNPCs were expanded as free floating neurospheres, see Figure 3, in defined DMEM/F12 medium supplemented with 2.0 mM L-glutamine, 0.6% glucose, N2-supplement, 2.0 µg/ml heparin, 20 ng/ml hbFGF, 20 ng/ml hEGF and 10 ng/ml hLIF. The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 and fresh media was added to the cultures every third day.

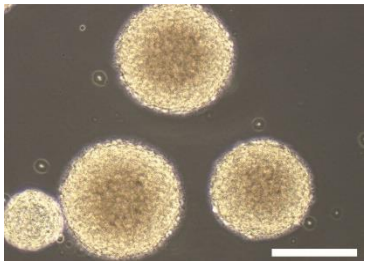

Figure 3. *Image of hNPCs as both free-floating neurospheres (aggregates) and single cells before dissociation. Image taken with a phase-contrast microscope. Scale bar: 200 µm.*

Before seeding, all cell culture surfaces (MEA plates and glass cover slips) were pre-coated with 10 µg/ml PLL solution for 1h at room temperature (RT) before washing with sterile water. The cell culture surfaces were then air-dried. Before coating, all MEA plates were cleaned with 1% Terg-A-Zyme solution (diluted in distilled water) and then rinsed with distilled water. 70% ethanol was then used for further sterilization. The neurospheres were sub-cultured by using mechanical dissociation and reseeded as single cells in differentiation medium (DMEM/F12 medium supplemented with 2.0 mM L-glutamine, 0.6% glucose, N2-supplement, 2.0 µg/ml heparin and 1% fetal bovine serum) at cell densities of approximately $1x10^4$ cells/cm^2 on each cell culture surface (30 000 cells/sample). Cell count and viability was assessed by trypan blue exclusion using an automated cell counter (TC20, BioRAD). hNPC cultures at 10 to 15 rounds of passaging were used.

Thawing and Plating of iCell® Neurons

The human induced pluripotent stem cell (hiPSC)-derived neurons used in this study were iCell® Neurons (Cellular Dynamics International, Madison, WI, USA). iCell® Neurons are a highly pure mixture of post-mitotic neural subtypes, comprised primarily of GABAergic (inhibitory) and glutamatergic (excitatory) neurons. Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 in an incubator. All cell culture surfaces (MEA plates, glass cover slips and chamber slides) were pre-coated with 0.1% PEI solution one day before thawing and plating of the iCell® Neurons. The solution was prepared by diluting 50% PEI solution in borate buffer (24 mM sodium tetraborate/50 mM boric acid in distilled water with pH adjusted to 8.4). Before coating, all MEA plates were cleaned with 1% Terg-A-Zyme solution (diluted in distilled water) and then rinsed with distilled water. 70% ethanol was then used for further sterilization. The iCell® Neurons were thawed in Complete iCell® Neurons Maintenance Medium supplemented with 2% iCell® Neurons Medium Supplement, 1% laminin (10µg/ml) and 1% penicillin-streptomycin. Cell count and viability was assessed by trypan blue exclusion using an automated cell counter (TC20, BioRAD).

For cell seeding on glass cover slips and chamber slides, the cell suspension was diluted in Complete iCell® Neurons Maintenance Medium supplemented with 2% iCell® Neurons Medium Supplement, 1% laminin (10µg/ml) and 1% penicillin-streptomycin. Cells were seeded with a cell density of 1×10^5 cells/cm^2 and the cell culture surfaces were then placed in a humidified incubator at 37°C and 5% CO_2.

For the study with the microelectrode arrays (MEAs), the cells were seeded directly over the electrode field of each MEA plate. The cells were seeded as 4 µl droplets of concentrated cell suspension with a cell density of approximately 73 000 cells/droplet, corresponding to a cell density of 3.7×10^6 cells/cm^2. This was done in order to maximize cell density on the electrode field. Next, the cells on the MEA plates were allowed to adhere for 40 minutes in a humidified incubator at 37°C and 5% CO_2. Before placing the MEA plates in an incubator, the MEAs were placed in plastic petri dishes and sterile water was added to the surrounding area (not the MEA chamber) to prevent droplet evaporation. The MEAs were also covered with sterile lids. After incubation, 900 µl of Complete iCell® Neurons Maintenance Medium supplemented with 2% iCell® Neurons Medium Supplement, 1% laminin (10µg/ml) and 1% penicillin-streptomycin was added to the MEAs. The MEAs were then placed back into the incubator.

After 1 day *in vitro* (1 DIV), 100% of the medium (cells should not be in contact with air) was replaced on all cell culture surfaces by filtered (0.22 µm filter) NBA medium supplemented with 10% KSR and 1% penicillin-streptomycin. After 5 DIV, 50% of the medium was changed and hereafter medium changes took place every third day.

Nanoparticles

The gold and silver nanoparticles (AuNPs and AgNPs) of both 20 and 80 nm in diameter used in this study were citrate stabilized colloidal NPs in water. The AuNPs and AgNPs were purchased from BBI International (Cardiff, UK). The NPs have been characterized and it has been concluded that the size and surface properties of the particles are in line with those reported by the manufacturer (Soderstjerna et al., 2014). NPs were added to cell cultures in low concentrations. The final concentration was 800 particles/cell in all NP treated samples, a particle concentration that is considered low (Soderstjerna et al., 2013).

Microelectrode Arrays

The three different types of microelectrode arrays (MEAs) used in this study were obtained from Multi Channel Systems MCS GmBH (Reutlingen, Germany). The MEA types have 60 titanium nitride (TiN) electrodes in a rectangular array with a diameter of either 10 or 30 µm. The electrode spacing is 200 µm. One MEA type (10 µm electrodes) is transparent since the contact pads and tracks are made of indium tin oxide (ITO) instead of titanium nitride (TiN). For the MEA types with contact pads and tracks opaque (TiN) the electrode diameter is either 10 or 30 µm. For more information about the different types of MEAs see Table 1 in appendix A. Cells are seeded onto the electrode grid inside the glass ring, see Figure 4.

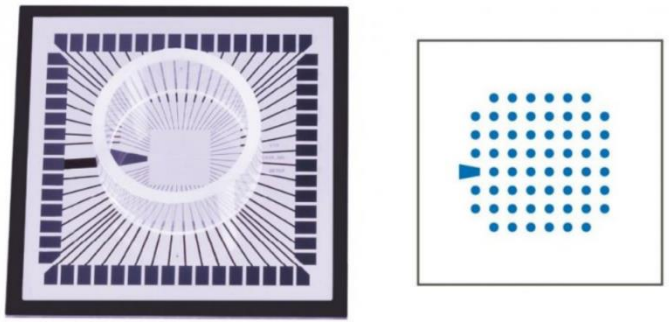

Figure 4. *MEA plate (49 mm x 49 mm) with contact pads and tracks opaque (left). Cells are seeded into the glass ring. Layout of the electrode grid inside the glass ring with 59 recording electrodes and 1 internal reference electrode (right) Electrode spacing is 200 µm.*

Experimental Setup for MEA Recordings

Spontaneous network activity was recorded at 37°C by placing the MEA plates in a filter preamplifier for 60 electrode MEAs (MEA 1060-Inv-BC) connected to a 60-channel filter amplifier (FA60SBC) and a temperature controller (TC02), see Figure 5. The whole experimental setup was obtained from Multi Channel Systems MCS GmbH (Reutlingen, Germany). The amplifier was connected to a PC on which data acquisition was managed with MC_Rack, a data acquisition and analysis software from Multi Channel Systems MCS GmbH (Reutlingen, Germany). MEAs were allowed to equilibrate for ~5 min prior to the 1 min recording of spontaneous activity. Raw data files were obtained by sampling the channels simultaneously with a gain of 1100x and a sampling frequency of 25kHz/channel. A Faraday cage was used in order to improve the signal-to-noise ratio (SNR) by blocking external electrical fields.

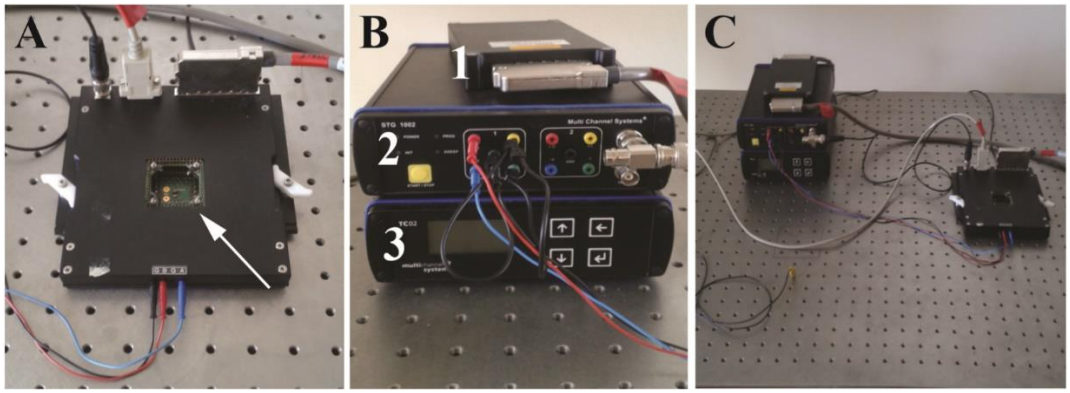

Figure 5. *Experimental setup for MEA recordings.* ***(A)*** *Preamplifier in which the MEA plate is placed. The arrow indicates the location of the MEA plate (currently occupied by a test MEA).* ***(B) 1.*** *Amplifier connected to the preamplifier and a PC.* ***2.*** *Stimulus generator (not used in this study).* ***3.*** *Temperature controller connected to the preamplifier.* ***(C)*** *The whole experimental setup with all parts visible.*

MEA Recordings of hNPCs

All three MEA types were used for initial MEA recordings of hNPCs. As previously mentioned, the hNPCs were seeded with a cell density of $1x10^4$ cells/cm^2 (30 000 cells/MEA) on PLL coated MEAs. 1 min recordings of spontaneous activity were done after 14 DIV (40% of MEAs) and after 21 DIV (60% of MEAs). The recordings were repeated every day until 28 DIV. MEAs with 10 µm electrodes and contact pads and tracks opaque were then used for further MEA trials since they provided the best SNR. In the following MEA studies, 1 min recordings of spontaneous activity started after 14 DIV and were repeated every day until 28 DIV. (After 15 DIV, gold and silver nanoparticles (AuNPs and AgNPs) with 80 nm in diameter were added for a final concentration of 800 particles/cell.)

MEA Recordings of iCell® Neurons

MEAs with 10 µm electrodes and contact pads and tracks opaque were used for recording activity from the iCell® Neurons. As previously mentioned, the cells were seeded as droplets containing approximately 73 000 cells/droplet (3.7×10^6 cells/cm^2) directly onto the PEI coated electrode field. 1 min recordings of spontaneous activity were done after 8 DIV, 9 DIV, 11 DIV, 13 DIV and then every day until 20 DIV. The recordings were made in an earlier stage than with the hNPCs according to instructions from the supplier (Cellular Dynamics International, Madison, WI, USA). After 13 DIV, gold and silver nanoparticles (AuNPs and AgNPs) with 80 nm in diameter were added for a final concentration of 800 particles/cell.

Data Analysis

After recording spontaneous activity, raw data files from the MC_Rack software were imported into MATLAB® (MathWorks, Natick, MA, USA) for digital filtering using a Butterworth band-pass filter with cutoff frequencies at 300 and 8000 Hz. The MATLAB® files were then imported into Offline Sorter (Plexon, Dallas, TX, USA) for further analysis. Offline Sorter is used for viewing and classifying action potential waveforms (spikes) collected from single electrodes. Spikes are displayed as points in a three-dimensional feature space where clustering techniques can be applied to sort the spikes.

Principal components analysis (PCA) is a useful technique for sorting spikes into clusters. By using PCA, the software chooses three principal components in the detected waveforms that provide the most reliable spike sorting. For detecting spikes in Offline Sorter, the threshold values were set to -16 µV and 7 µV, meaning that anything above or below the thresholds can be regarded as a spike.

Scanning Electron Microscopy

For scanning electron microscopy (SEM), the hNPCs and iCell® Neurons were fixed in a solution of 4% paraformaldehyde (PFA) in 200 mM Sørensen's buffer (200 mM KH_2PO_4, 200 mM Na_2HPO_4) for 10 min at RT and washed three times with phosphate buffered saline (1xPBS), pH 7.2. The cell cultures were then dehydrated in a series of ethanol with increasing concentrations, *i.e.* 50%, 70%, 85%, 95% and 100%, prior to critical point drying (CPD030, Leica Microsystems GmbH, Kista, Sweden). The glass cover slips with cells were then mounted on metal stubs. Gold-palladium (~15 nm) was used to sputter-coat (Cressington Sputter Coater 108 auto, Cressington Scientific Instruments, Watford, UK) the cell samples before imaging in a SEM (SU3500, Hitachi, Tokyo, Japan) at an accelerating voltage of 5 kV.

Imaging of hNPCs with Scanning Electron Microscopy

As previously mentioned, hNPCs were seeded on PLL coated glass cover slips with a cell density of 1×10^4 cells/cm^2. Cell samples were fixed in PFA after 9, 16 and 23 DIV for SEM-studies regarding the morphology of the hNPCs. No nanoparticles were added to the hNPCs seeded on glass cover slips for SEM.

Imaging of iCell® Neurons with Scanning Electron Microscopy

iCell® Neurons were seeded on PEI coated glass cover slips with a cell density of 1×10^5 cells/cm^2 for SEM. No SEM-studies regarding persistent or acute NP exposure were done due to a limited number of cells. The chamber slides were fixed in PFA after 10 DIV. iCell® Neurons seeded on a MEA plate were also prepared for SEM-studies. The cells were seeded as droplets containing approximately 73 000 cells/droplet (3.7×10^6 cells/cm^2) directly onto the electrode field. The cells on the MEA plate were fixed in PFA after 20 DIV and the MEA plate was then cut into smaller pieces in order to fit into the SEM.

Immunocytochemistry

Before immunocytochemical (ICC) staining the hNPCs and iCell® Neurons were fixed in a solution of 4% paraformaldehyde (PFA) in 200 mM Sørensen's buffer (200 mM KH_2PO_4, 200 mM Na_2HPO_4) for 10 min at RT and washed three times with phosphate-buffered saline (1xPBS), pH 7.2. The cultures were blocked and permeabilized using a solution of PBS, 1% bovine serum albumin and 0.25% Triton X-100 for 30 min. Thereafter, the cell cultures were incubated with primary antibodies diluted in blocking solution for at least 16h at 4°C. Then, after removing the primary antibody solution and washing with PBS, the cells were incubated with secondary antibodies diluted in blocking solution for 1h at RT in the dark. The cell samples were then mounted on glass slides using Vectashield mounting medium containing antifading medium and DAPI for staining of cell nuclei. The glass slides were stored at -20°C. Full list of all the primary and secondary antibodies used are available in Table 2, 3 and 4. The glass slides where then analyzed with a fluorescence microscope (Axio Imager M2, Carl Zeiss, Germany) while some glass slides were analyzed further using a confocal microscope (Zeiss LSM 510 META, Carl Zeiss, Germany).

Immunocytochemical Staining of hNPCs

As previously mentioned, hNPCs were seeded on PLL coated glass cover slips with a cell density of 1×10^4 cells/cm^2. For ICC-studies regarding persistent and acute NP exposure, four different NP treatments were used, see Table 1. The NPs added to the cell cultures were AuNPs (20 nm in diameter), AuNPs (80 nm in diameter), AgNPs (20 nm in diameter) and AgNPs (80 nm in diameter). The NPs were added to reach a final concentration of 800 particles/cell. Control samples were not exposed to NPs. For persistent treatments, NPs were added after two days of acclimatization and cell samples were fixed in PFA after 9 DIV (7 days with treatment), 16 DIV (14 days with treatment) and 23 DIV (21 days with treatment). For acute treatment (exposure for 24h), NPs were added after 15 DIV and fixed in PFA the following day (16 DIV). Two separate cell seedings were done and six samples for each treatment group (n=6) were analyzed. Primary antibodies used for ICC with hNPCs are visible in Table 2.

Table 1. *Experimental overview of the study with acute (24h) and persistent (7, 14 and 21 days) exposure to gold nanoparticles (AuNPs) and silver nanoparticles (AgNPs) with different diameters (20 or 80 nm).*

24h 7 days 14 days 21 days	24h 7 days 14 days 21 days	24h 7 days 14 days 21 days	24h 7 days 14 days 21 days	24h 7 days 14 days 21 days

Immunocytochemical Staining of iCell® Neurons

iCell® Neurons were seeded on PEI coated 8 well chamber slides with a cell density of 1×10^5 cells/cm^2 for ICC. No NPs were added to the chamber slides due to a limited number of cells. The chamber slides were fixed in PFA after 10 DIV. Primary antibodies used for ICC with iCell® Neurons are visible in Table 2 and Table 3.

Table 2. *Primary antibodies used for immunocytochemistry. Antibodies used for studying neurons in different stages of differentiation and for visualizing astrocytes.*

Antigen	Host	Target Cell	Dilution	Source	Cat #
Nestin	Rabbit	Progenitor cells[1]	1:1500	Millipore, Temecula, CA, USA	ABD69
Doublecortin	Goat	Immature neurons[2]	1:200	Santa Cruz Biotechnology, Inc., Dallas, TX, USA	sc-8066
β-tubulin III	Mouse	Early neurons[3]	1:1500	Sigma-Aldrich, St Louis, MO, USA	T8660
MAP2B	Mouse	Mature neurons[4]	1:200	Sigma-Aldrich, St Louis, MO, USA	M1406
GFAP	Rabbit	Astrocytes[5]	1:2000	DAKO A/S, Glostrup, Denmark	Z0334

1. (Messam et al., 2000; Englund et al., 2002), 2. (Piens et al., 2010) 3. (Englund et al., 2002) 4. (Kalcheva et al., 1998) 5. (van Bodegraven et al., 2019)

Table 3. *Primary antibodies used for immunocytochemistry. Antibodies used for studying neuronal synapses. Synaptophysin, GABA and vGluT-2 are pre-synaptic markers whereas gephyrin and PSD95 are post-synaptic markers.*

Antigen	Host	Target Cell	Dilution	Source	Cat #
Synaptophysin	Rabbit	Neuronal synapses[1]	1:500	Abcam, Cambridge, MA, USA	ab32594
GABA	Mouse	GABAergic neurons (inhibitory)[2]	1:250	Sigma-Aldrich, St Louis, MO, USA	A0310
vGluT-2	Rabbit	Glutamatergic neurons (excitatory)[3]	1:500	Synaptic Systems GmbH, Göttingen, Germany	135403
Gephyrin	Mouse	GABAergic neurons (inhibitory)[4]	1:3000	Synaptic Systems GmbH, Göttingen, Germany	147011
PSD95	Mouse	Postsynaptic elements (excitatory)[5]	1:200	Abcam, Cambridge, MA, USA	ab2723

1. (Thiel, 1993; Harper et al., 2017) 2. (Gonchar et al., 2007; Achim et al., 2014) 3. (Takamori et al., 2001) 4. (Brady and Jacob, 2015) 5. (Coley and Gao, 2018; Yu et al., 2018)

Table 4. *Secondary antibodies used for immunocytochemistry.*

Species	**Target**	**Fluorochrome**	**Dilution**	**Source**	**Cat #**
Donkey	Anti-Goat	Alexa Fluor 488	1:200	Molecular Probes, Inc., Eugene, OR, USA	A11055
Donkey	Anti-Goat	Texas Red	1:100	Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA	705-076-147
Donkey	Anti-Mouse	Alexa Fluor 488	1:400	Molecular Probes, Inc., Eugene, OR, USA	A21202
Donkey	Anti-Rabbit	Texas Red	1:200	Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA	711-076-152

Results

Human Neural Progenitor Cells

Immunocytochemistry (hNPCs)

The hNPCs differentiate *in vitro* into the three main phenotypes of the CNS, *i.e.* neurons, astrocytes and oligodendrocytes. Nestin, a marker for progenitor cells, and doublecortin, a marker for immature neurons, were used in order to evaluate neuronal differentiation in the cell cultures. Nestin and doublecortin were detected after 16 DIV (Figure 6). Nestin was expressed by the cells to a higher extent than doublecortin. Doublecortin expression was mostly found in nestin positive cells. Some neurons stained with doublecortin displayed complex morphologies with extended neuronal processes whereas others showed a low level of maturation (no neuronal processes) after 16 DIV.

The quantitative analysis of hNPCs exposed to AgNPs and AuNPs in low concentrations in order to evaluate if NPs affect neuronal differentiation is not included in this thesis. The analysis regarding both acute and persistent NP treatment was instead made by Axel Karlsson and Isabel Ulmert as a part of their bachelor's thesis.

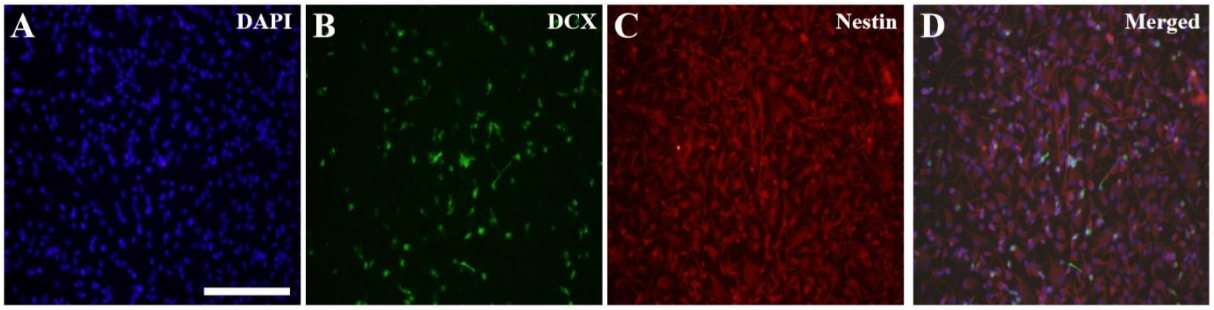

Figure 6. *Representative images of immunocytochemical staining of hNPCs after 16 DIV. Nestin (progenitor cells) was extensively detected in the cell cultures (**C**). DCX (doublecortin), a marker for immature neurons, was detected (**B**) but to a lesser extent and with fewer complex morphologies compared to the nestin positive cells in **C**. Cell nuclei were stained with DAPI (**A**). Scale bar in **A**: 200 µm.*

Further immunocytochemical analysis with doublecortin shows that the neurons develop complex neuronal morphologies after 23 DIV (Figure 7). Some complex morphologies are seen after 16 DIV whereas only a low rate of development is seen in the hNPC cultures after 9 DIV. The low rate of development is indicated by the fact that only one neuronal process or no processes at all are displayed by the vast majority of the cells.

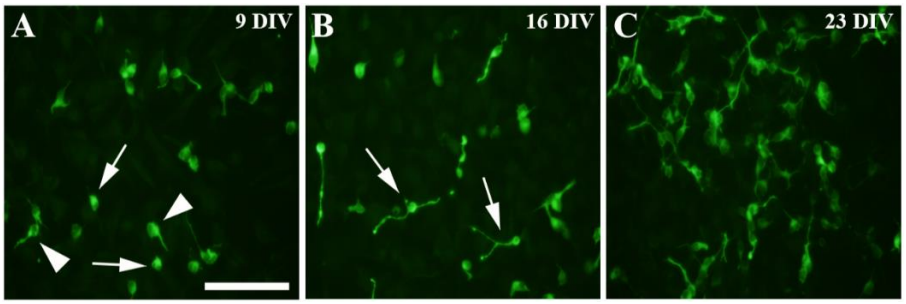

Figure 7. *Representative images of immunocytochemical staining of hNPCs with doublecortin, a marker for immature neurons, after 9 DIV (**A**), 16 DIV (**B**) and 23 DIV (**C**). The neurons mostly display a low rate of development with no extended processes (arrows in **A**) or one extended process (arrowheads in **A**) after 9 DIV. More complex morphologies (arrows in **B**) are seen after 16 DIV whereas neuronal networks are starting to develop after 23 DIV. Scale bar in **A**: 100 µm.*

Scanning Electron Microscopy (hNPCs)

SEM images were taken of hNPCs on glass cover slips after 9, 16 and 23 DIV in order to study the morphological development of neurons and glial cells (Figure 8). Glial cells develop a confluent layer beneath the neuronal cells. This is first observed after 9 DIV and seen in all SEM images containing hNPCs regardless of the time the cell cultures have spent *in vitro*. The neuronal cells only show a low rate of development in the SEM images after 9 DIV, however, maturation is seen after 16 DIV since the neurons have an increased amount of extended processes. The neuronal development has progressed further after 23 DIV, this is indicated by more complex neuronal morphologies.

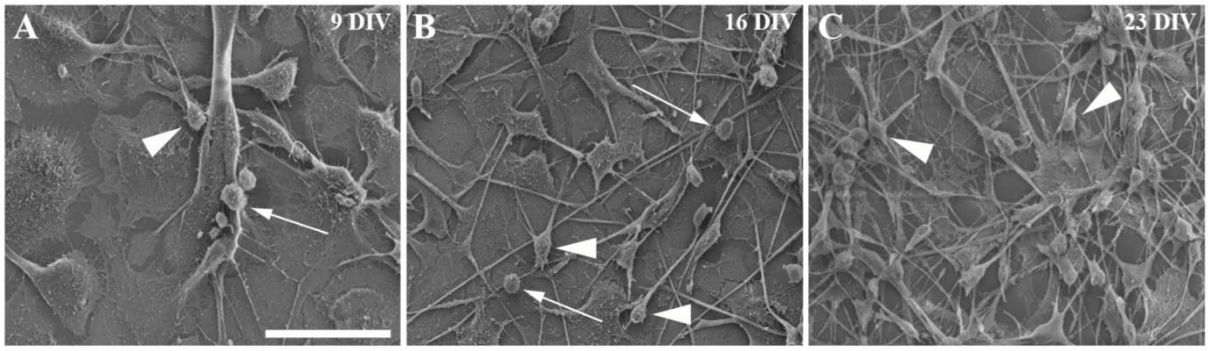

Figure 8. *Representative SEM images of hNPCs cultured on glass cover slips. The images were taken after 9 DIV (**A**), 16 DIV (**B**) and 23 DIV (**C**). Arrows indicate neurons with a low rate of development whereas arrowheads indicate neurons with one or more extended processes. Glial cells form a confluent layer beneath the neurons after 9 DIV. Maturation of neurons is seen with extended neuronal processes and more complex morphologies after 16 and 23 DIV. Scale bar in **A**: 50 µm. Magnification: 800x.*

MEA Recordings (hNPCs)

The hNPCs survived the seeding onto the MEA plates and attached to the electrode grid (Figure 9). However, no significant extracellular activity was recorded from *in vitro* cultured hNPCs on MEA plates. Several one minute recordings were made of hNPCs seeded on MEAs in independent trials with a cell density of 1×10^4 cells/cm^2. Recordings were made with all three MEA types but mainly with the MEAs with 10 µm electrodes and contact pads and tracks opaque. Recordings were started after 14 DIV and continued every day until 28 DIV.

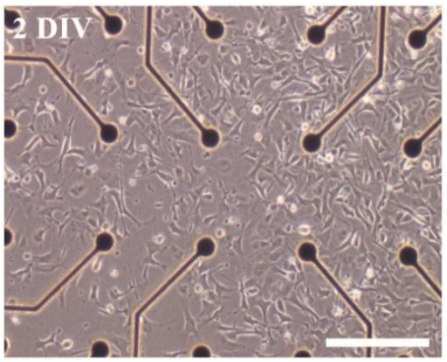

Figure 9. *hNPCs on a MEA plate after 2 DIV. The cells have survived and attached to the electrode grid on the MEA plate. Scale bar: 200 µm. Image taken with a phase-contrast microscope.*

<u>Human Induced Pluripotent Stem Cell-Derived Neurons</u>

Immunocytochemistry (iCell® Neurons)

The iCell® Neurons are a highly pure mixture of neural subtypes according to supplier. This was evaluated by staining the cells for GFAP (glial fibrillary acidic protein), a cell-specific marker for astrocytes in the developing CNS. GFAP was not detected after 10 DIV (Figure 10). Markers for progenitor cells (nestin) and neuronal cells in different maturation stages (doublecortin, β-tubulin III and MAP2B) were used in order to evaluate if the iCell® Neurons form mature neuronal networks with differentiated cells (Figure 10). Nestin, an intermediate filament protein associated with progenitor cells in the CNS, was detected in the cell cultures. Doublecortin, a microtubule stabilizing protein associated with immature neurons, was detected in nestin negative cells as well as double-labeled with nestin. β-tubulin III, a marker for early neurons, was detected after 10 DIV as well. β-tubulin III was found co-localized with nestin and in nestin negative cells. MAP2B (microtubule-associated protein 2B), a marker for mature neurons as well as neurons in a developing state, was also detected in the neuronal cultures. Even though nestin was detected in the neuronal cultures it was expressed to a lesser extent than the markers for differentiating neurons. The immunocytochemical study with markers for differentiating neurons also shows that the iCell® Neurons develop complex morphologies and neuronal networks after 10 DIV (Figure 10).

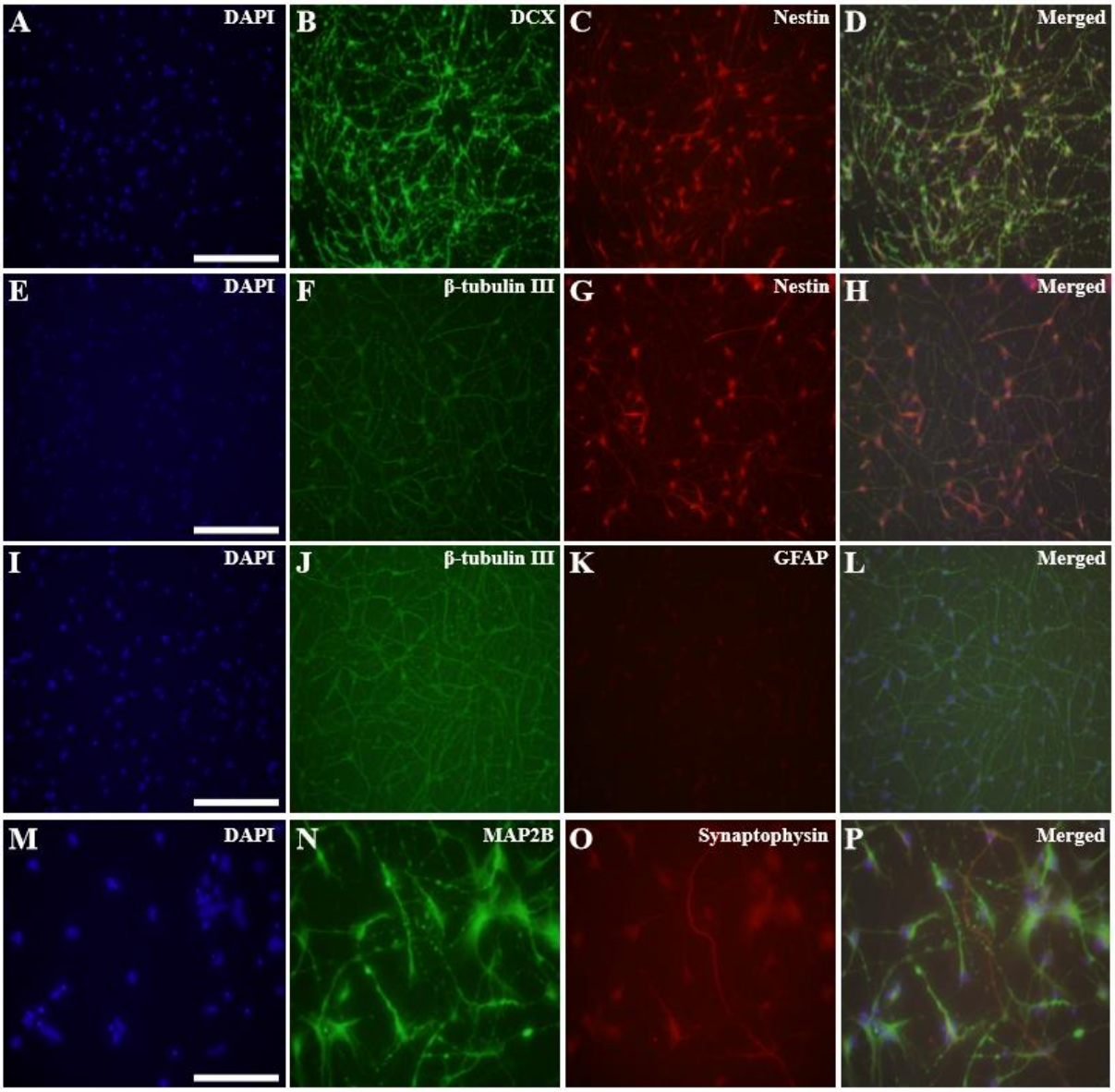

Figure 10. *Representative images of immunocytochemical staining of iCell® Neurons after 10 DIV. Nestin (progenitor cells) was detected in the cell cultures (**C** and **G**). DCX (doublecortin) (**B**), a marker for immature neurons, was detected in cells with both positive and negative expression of nestin. β-tubulin III (**F** and **J**), a marker for early neurons, was also co-localized with nestin and expressed by nestin negative cells. Positive expression of MAP2B (mature neurons) was detected (**N**). Negative expression of GFAP (**K**) indicates that no cells have differentiated into glial cells. Positive expression of synaptophysin (neuronal synapses) was also detected (**O**). Cell nuclei are stained with DAPI (**A**, **E**, **I** and **M**). Scale bar in **A**, **E** and **I**: 200 µm. Scale bar in **M**: 100 µm.*

The iCell® Neurons are a mixture of neural subtypes, comprised primarily of GABAergic (inhibitory) and glutamatergic (excitatory) neurons. In order to evaluate if the iCell® Neurons form functional neuronal networks with both GABAergic and glutamatergic neurons the presence of both pre- and post-synaptic proteins in inhibitory and excitatory synapses was studied as well as markers for neuronal synapses (Figure 11). As mentioned before, the cell cultures were fixed after 10 DIV. Expression of synaptophysin, a membrane protein of synaptic vesicles and thus a marker for neuronal synapses, was detected in cells with both negative and positive expression of β-tubulin III.

Post-synaptic markers associated with both GABAergic and glutamatergic neurons were found. PSD95 (post-synaptic density 95), a marker for post-synaptic NMDA receptors in excitatory neurons, was found double-labeled with doublecortin and detected in doublecortin negative cells. Gephyrin, a marker for post-synaptic neurotransmitter receptors in inhibitory neurons, was mostly detected in doublecortin positive cells. Pre-synaptic markers associated with both GABAergic and glutamatergic neurons were found. However, the immunostainings were not as convincing as the ones involving post-synaptic markers. GABA (γ-aminobutyric acid), an inhibitory neurotransmitter, seems to be detected in the neuronal cultures. vGluT-2 (vesicular glutamate transporter 2), a marker for proteins transporting the excitatory neurotransmitter glutamate, also appears to be detected but very weakly.

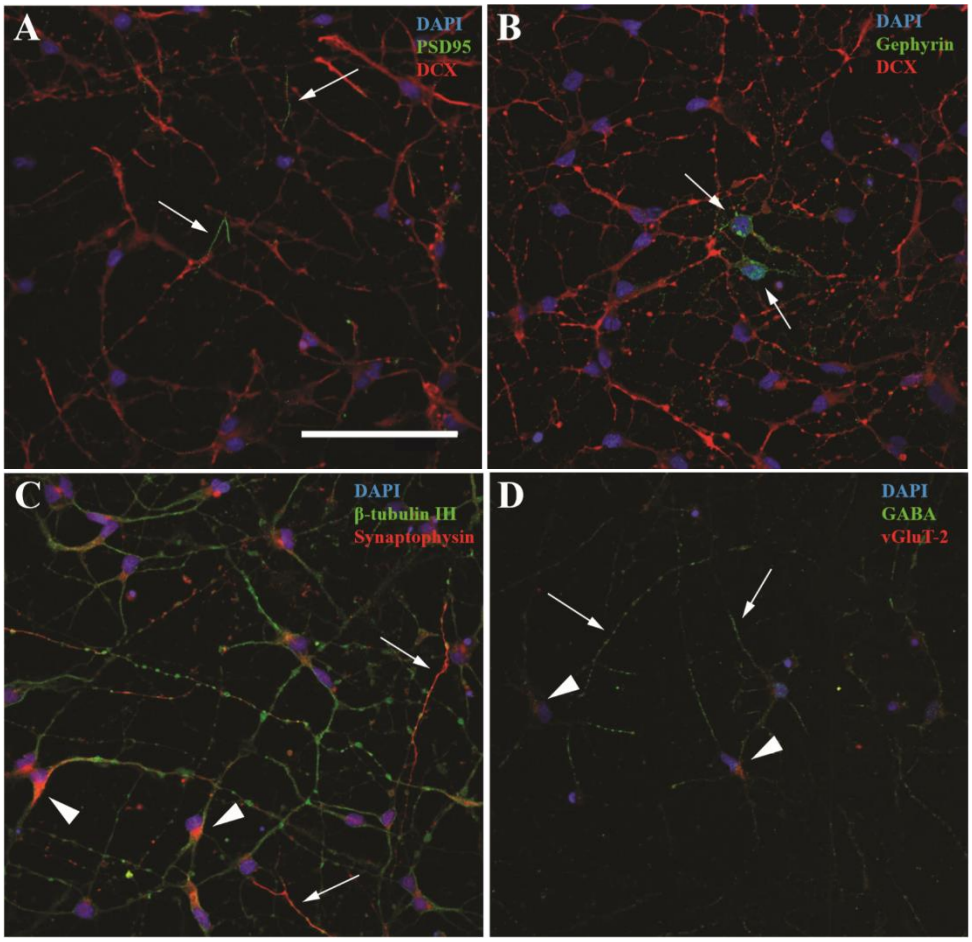

Figure 11. *Representative images of immunocytochemical staining of pre- and post-synaptic markers as well as markers for neuronal synapses in iCell® Neurons after 10 DIV. Excitatory post-synaptic marker PSD95 was detected (arrows in* ***A****) in the neuronal cultures as well as the inhibitory post-synaptic marker gephyrin (arrows in* ***B****). Synaptophysin, a marker for neuronal synapses, was found co-localized with β-tubulin III (arrowheads in* ***C****) and in β-tubulin III negative cells (arrows in* ***C****). Inhibitory neurotransmitter GABA seems to be detected (arrows in* ***D****) whereas excitatory pre-synaptic marker vGluT-2 is only weakly stained (arrowheads in* ***D****). Cell nuclei are stained with DAPI (blue). Scale bar in* ***A****: 100 µm. Images taken with a confocal microscope.*

Scanning Electron Microscopy (iCell® Neurons)

SEM images of iCell® Neurons seeded on glass cover slips and fixed after 10 DIV shows that the cells have formed complex morphologies with several processes extending from the cell bodies (Figure 12). The cells seem to have formed neuronal networks as well as clusters, see Figure 12. The clusters contain numerous cells and vary in size. SEM images of neurons cultured directly onto the electrode grid of MEA plates and fixed after 20 DIV have attached themselves to the 10 µm electrodes (Figure 12). The fact that the cells are located near or on top of electrodes provides a good basis for electrophysiological recordings.

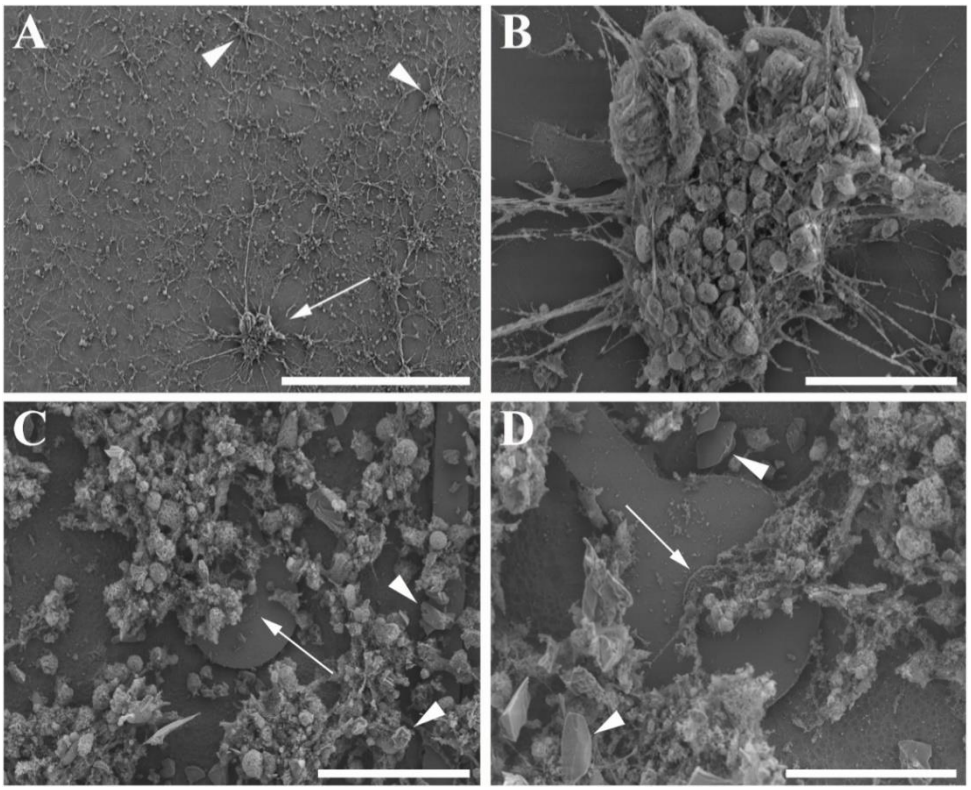

Figure 12. *Representative SEM images of iCell® Neurons on glass cover slips after 10 DIV (**A** and **B**) and on MEA plates after 20 DIV (**C** and **D**). The cell cultures form neuronal networks with complex morphologies after 10 DIV. Clusters of cells are visible (arrowheads and arrow in **A**). The cluster indicated by the arrow in **A** is further enlarged in **B**. The neurons have survived and attached to electrodes (arrow in **C** and **D**) on the MEA plates. Glass fragments from cutting the MEA plates into smaller pieces after fixation are visible in the images (arrowheads in **C** and **D**). Scale bar in **A**: 500 µm. Scale bar in **B** and **C**: 50 µm. Scale bar in **D**: 30 µm.*

MEA Recordings (iCell® Neurons)

In order to study if gold and silver nanoparticles affect neuronal function, recordings of electrical activity were made with MEAs. Significant extracellular activity on MEAs was observed from iCell® Neurons on the first day of recording (8 DIV). Raw data recordings with spontaneous action potential waveforms (spikes) and local field potentials are seen in Figure 13. Spontaneus neuronal activity was appearent on more electrodes after 11 days and the activity seemed to peak after 13 DIV. Extracellular activity was observed on MEAs during every recording session until the 60 second recordings were stopped after 20 DIV. Both small positive spikes and large negative spikes were recorded.

However, only approximately 10% of the electrodes showed signs of significant extracellular activity whereas the remaning 90% only displayed local field potentials. This did not provide a good basis for quantitative studies of the effect of nanoparticles on neuronal function. AuNPs and AgNPs with a diameter of 80 nm were added after 13 DIV in low concentrations (800 particles/cell). Due to a low number of active electrodes no quantitative analysis was done. A qualitative analysis of neurons exposed to AuNPs is seen in Figure 13. The amplitude of the recorded waveforms decreased after addition of AuNPs and the activity dissapeared after four days of exposure. The electrical activity on the control MEA did not dissapear completely after 20 DIV (Figure 13). This is however not a clear indication of neurotoxicity and the decreasing activity is most likely due to cell migration. The recordings with electrode 82 (MEA 7) in Figure 13 was analyzed further using principal components analysis (PCA) in Offline Sorter.

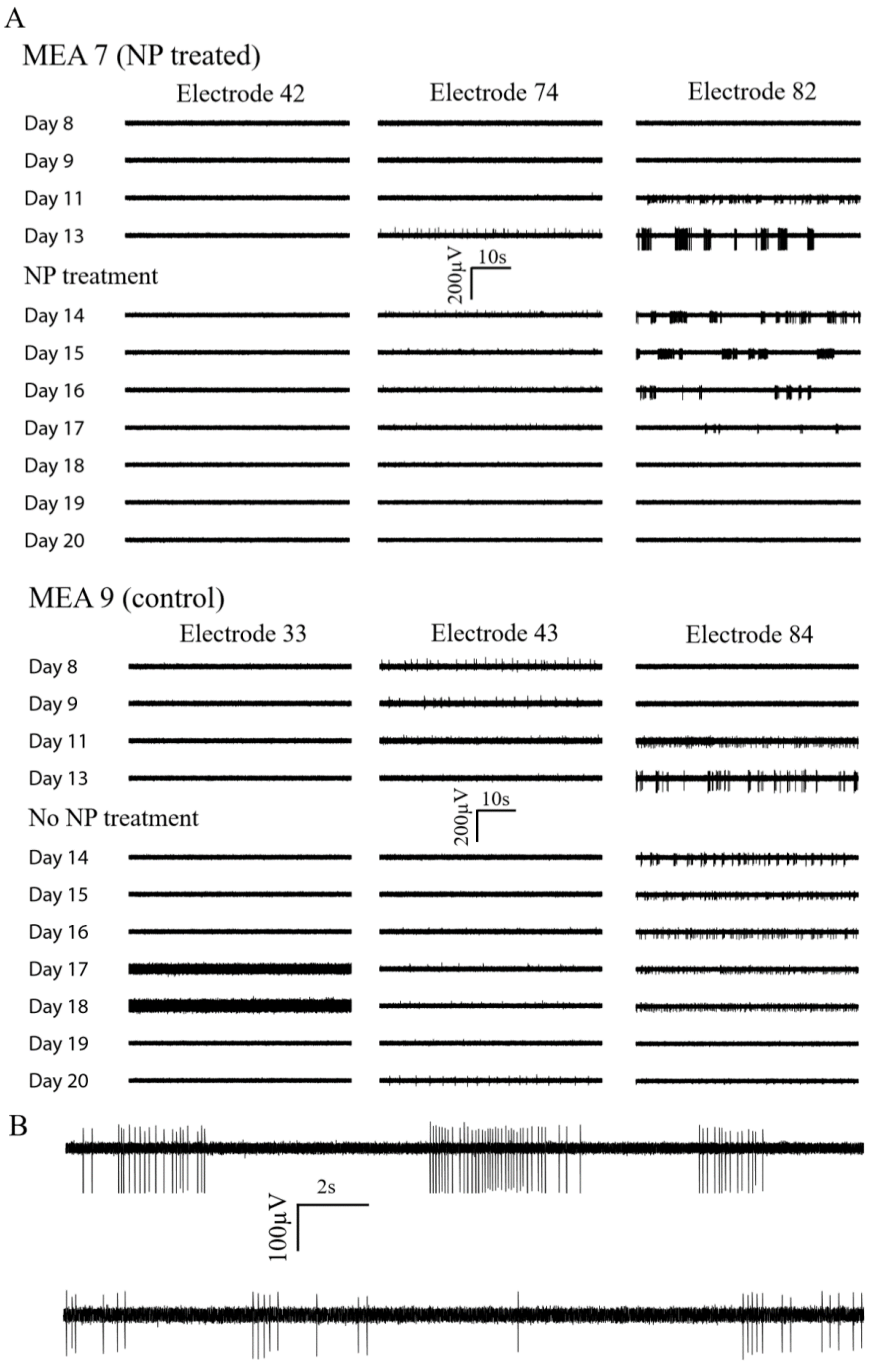

Figure 13. *Qualitative analysis of raw data recordings of spontaneous activity from iCell® Neurons. Recorded extracellular activity from three electrodes on two separate MEA plates during different days is displayed* ***(A)****. The time period is one minute. AuNPs (800 particles/cell) with a diameter of 80 nm was added to MEA 7 after 13 DIV. Electrode 33 (MEA 9) and electrode 42 (MEA 7) display no signs of significant electrical activity (that was the case for approximately 90% of the electrodes on all MEA plates). Electrode 43 (MEA 9) and electrode 74 (MEA 7) have clear positive action potential waveforms (spikes) whereas electrode 84 (MEA 9) and electrode 82 (MEA 7) display recordings of large negative spikes. The electrical activity on MEA 7 decreases after exposure to AuNPs and disspears after four days of NP treatment. The activity on MEA 9, no NP exposure, does not dissapear completely after 20 DIV.* ***(B)*** *10 second segments of electrical activity from iCell® Neurons after 13 DIV from electrode 82 on MEA 7 (upper figure) and electrode 84 on MEA 9 (lower figure). The recorded activity is typical for healthy neuronal networks.*

Recorded spikes from electrode 82 (MEA 7) in Figure 13 were sorted into clusters using PCA in Offline Sorter (Figure 14). This was done in order to get more information about the recorded electrical activity. The thresholds for spike sorting in Offline Sorter were set manually. Spontaneous electrical activity from at least four different neurons was recorded on the same electrode on day 13 since the spikes were sorted into four different clusters. Clear multiunit extracellular activity was in other words detected. This is further demonstrated by a waveform analysis of the sorted spikes (Figure 14). Spikes belonging to a specific cluster have similar waveforms. Waveforms differ from one cluster to another when it comes to amplitude, wavelength and other wave characteristics thus indicating that there are several different neurons in the vicinity of the electrode. The same electrode (electrode 82) did not display any clear spiking activity after 8 DIV and that data was therefore used as control when performing the analysis in Offline Sorter.

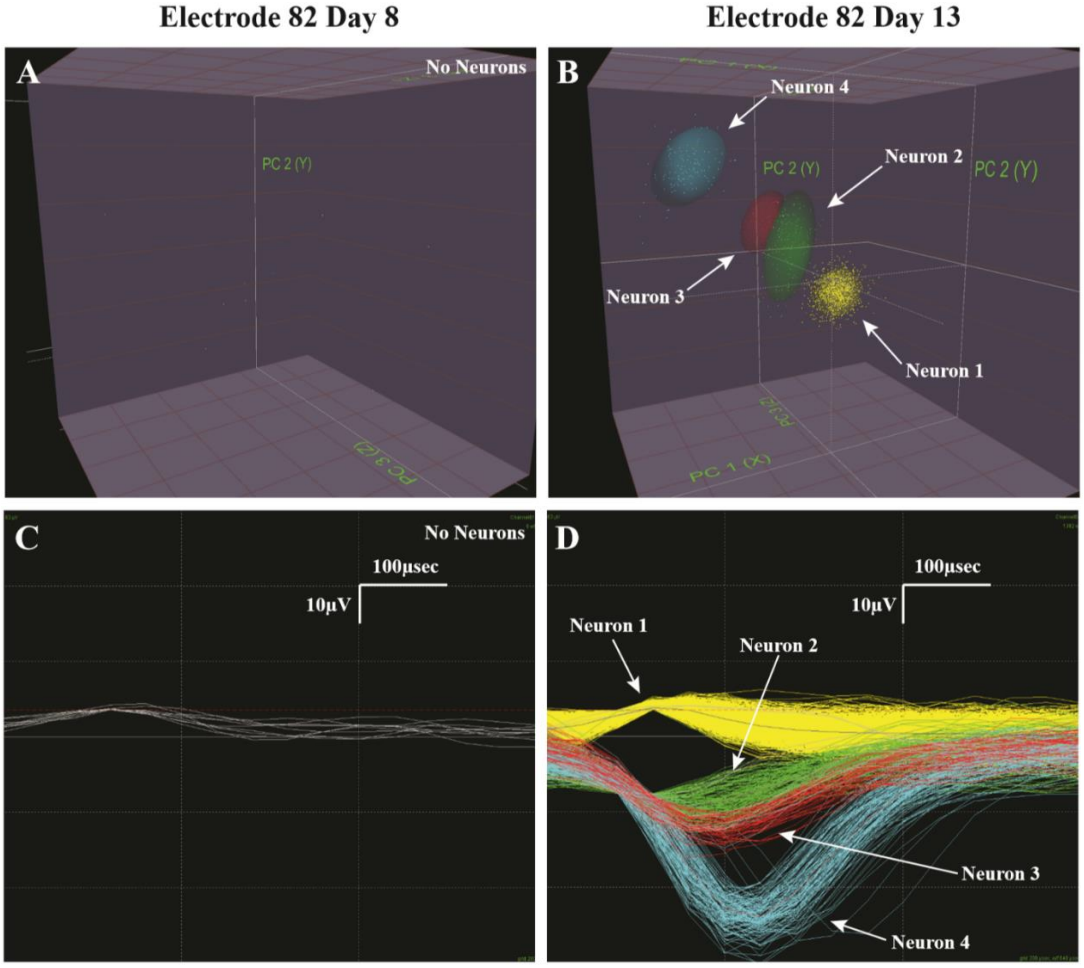

Figure 14. *Sorting of recorded action potential waveforms (spikes) from iCell® Neurons* ***(A and B)****. Spikes are sorted into clusters in Offline Sorter using PCA. Spikes are displayed as points in a three-dimensional space. Electrode 82 on MEA 7 after 8 DIV* ***(A)*** *displays a few recorded spikes but no clusters of spikes thus indicating an absence of significant electrical activity. Electrode 82 on MEA 7 after 13 DIV* ***(B)*** *shows four clusters of sorted spikes meaning that extracellular activity from four different neurons has been recorded on the same electrode.* ***(C)*** *Waveforms of recorded spikes from iCell® Neurons on electrode 82 on MEA 7 after 8 DIV.* ***(D)*** *Waveforms of sorted spikes from iCell® Neurons on electrode 82 after 13 DIV. The different waveforms indicate clear multiunit extracellular activity from four neurons. The color of each waveform* ***(D)*** *corresponds to the color of a cluster* ***(B)****. No NPs were added prior to these recordings.*

Recordings of iCell® Neurons during 6 second segments on electrode 82 after 8 DIV and 13 DIV are seen in Figure 15. As mentioned before, recordings after 8 DIV on electrode 82 shows no signs of significant extracellular activity and this data was therefore used as control. Recordings after 13 DIV displays that significant extracellular activity from four different neurons has been recorded. By comparing spikes from the filtered data set to the spikes registered as neuronal activity in Offline Sorter it is further demonstrated that the spiking activity is originating from several different neurons. For instance, the large negative spikes are sorted into one cluster wheareas the smaller positive spikes are sorted into another cluster.

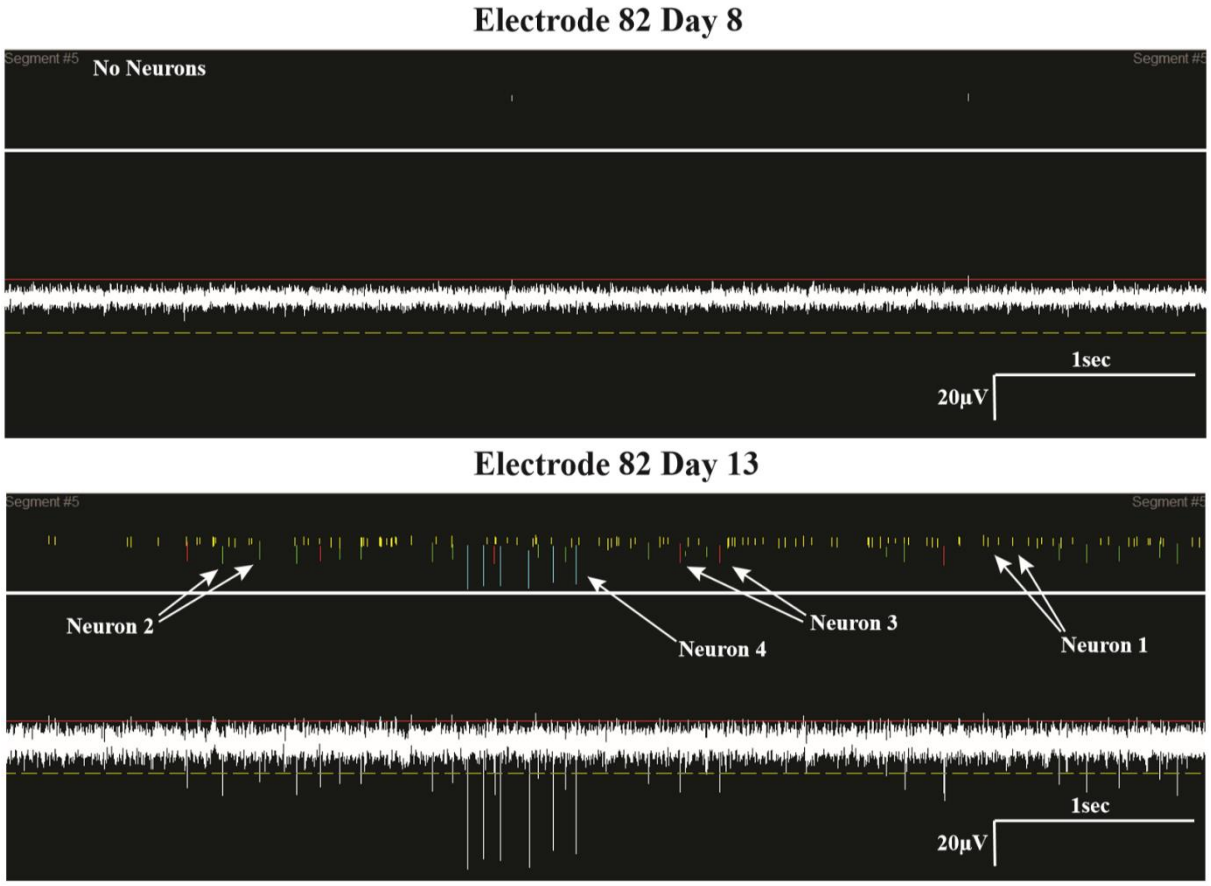

Figure 15. *Band-pass filtered recordings of iCell® Neurons during 6 second segments on electrode 82 after 8 DIV* ***(upper figure)*** *and 13 DIV* ***(lower figure)****. Recordings after 8 DIV show no signs of significant extracellular activity. However, there are a couple of events registered as spikes. Recordings after 13 DIV show significant extracellular activity registered on electrode 82. Spontaneous activity from four different neurons has been recorded and the color of each individual spike corresponds to a cluster in* ***Figure 14 (B)*** *and a waveform in* ***Figure 14 (D)****. Threshold values were set manually to 7µV (red line) and -16µV (yellow line).*

Discussion

Human Neural Progenitor Cells

No significant extracellular activity associated with action potential waveforms was recorded from hNPCs using MEA plates. The hNPCs differentiate *in vitro* into the three major cell types of the CNS, *i.e.* neurons, astrocytes and oligodendrocytes (Carpenter et al., 1999; Englund et al., 2002). Immunocytochemical staining with doublecortin, a marker for immature neurons, show that the neurons develop complex morphologies after 16 DIV and form neuronal networks after 23 DIV. However, nestin is still highly expressed after 16 DIV which indicates that a lot of cells remain in a progenitor like state. Immunocytochemical analysis of the same *in vitro* cultured hNPC type shows expression of markers for mature neurons as well as expression of neuronal pre- and post-synaptic inhibitory and excitatory markers after 20 DIV (Jakobsson et al., 2017) This indicates maturation into functional neurons and neuronal networks. Spontaneous action potential waveforms have been recorded from the same hNPC type (Jakobsson et al., 2017). This was done with hNPCs seeded in electrospun poly-ε-caprolactone (PCL) fiber scaffolds with single channel extracellular probes inserted into the fiber network. In other words, electrophysiological recordings of hNPCs should be a possibility with MEA plates as well.

Increasing cell density of hNPCs seeded on MEAs could provide better results. hNPCs were seeded on MEAs with a cell density of 1×10^4 cells/cm^2. The cell density was 9 times higher (9×10^4 cells/cm^2) for hNPCs seeded in PCL fiber scaffolds (Jakobsson et al., 2017) and 370 times higher (3.7×10^6 cells/cm^2) for iCell® Neurons seeded as droplets of concentrated cell suspension on MEAs.

SEM images of hNPCs shows that the cell cultures have a confluent glial cell carpet with neurons located on top. This layered appearance is typical for cells cultured on 2D surfaces (Gramowski et al., 2010) and could cause difficulties when recording electrical activity due to increased distance between electrodes and neurons. Seeding hNPCs in a 3D scaffold of electrospun PCL fibers is one approach of getting glial and neuronal cells more intermingled rather than layered (Jakobsson et al., 2017). Using 3D scaffolds would also result in a more *in vivo* like microenvironment since polymeric nanofibers can mimic biological entities such as the extracellular matrix (ECM) (Pelipenko et al., 2015). The ECM is important in neural development since it coordinates cell proliferation, migration and differentiation (Dityatev and Schachner, 2003). Another solution to the problem of having a glial cell layer between neurons and electrodes is to use MEAs with protruding 3D electrodes instead of planar electrodes. 3D electrodes allow tissue penetration in order to get closer to the active neurons (Heuschkel et al., 2002).

As previously mentioned, the hNPCs differentiate into glial cells as well as neurons. Increasing the amount of progenitor cells that differentiate into neuronal cells could enhance the probability of getting electrophysiological recordings with MEAs. This could be done by altering the differentiation medium in which the cells are cultured.

Human Induced Pluripotent Stem Cell-Derived Neurons

Detection of all markers used for identifying neurons in different stages of maturation (doublecortin, β-tubulin III and MAP2B) indicate that the iCell® Neurons develop into mature neuronal networks after 10 DIV. Nestin, a marker for progenitor cells, was detected as well but to a lesser extent indicating that some cells still remain in an undifferentiated state after 10 DIV. Detection of post-synaptic markers for inhibitory (gephyrin) and excitatory (PSD95) synapses as well as positive staining with synaptophysin (neuronal synapses) indicate that the iCell® Neurons form functional neuronal networks after 10 DIV. Further studies with the pre-synaptic markers GABA (inhibitory) and vGluT-2 (excitatory) might however be needed since the immunocytochemical study was unable to provide conclusive evidence of positive staining. GABA and vGluT-2 are likely to be expressed in the cell cultures since the post-synaptic markers for excitatory and inhibitory neurons were detected as well as the pre-synaptic marker synaptophysin. GFAP was not detected in the neuronal cultures after 10 DIV. GFAP is mainly expressed by astrocytes but can however be expressed by immature, nondifferentiated cells in the CNS (van Bodegraven et al., 2019). Further studies with GFAP might be needed since nestin, a marker for progenitor cells, was detected.

SEM images of iCell® Neurons cultured of MEA plates show that the cells have attached to the microelectrodes. This, combined with the results from the immunocytochemical analysis, provides a good basis for successful electrophysiological recordings. Significant multiunit extracellular activity was indeed recorded from the neuronal cultures on the MEAs. Spontaneous action potential waveforms from four different neurons recorded from a single microelectrode were visualized when sorting spikes using principal components analysis in Offline Sorter. Large negative spikes were recorded on the MEAs and that specific type of activity is associated with the perisomatic area of neurons (Obien et al., 2015). Small positive spikes were registered as well and that type of spiking activity is associated with the dendritic area of a neuron (Obien et al., 2015). However, the thresholds used for spike detection were set manually. This could cause difficulties when analyzing large data sets with neuronal activity recorded by a multitude of electrodes. The method for performing a cluster analysis needs to be improved but even though MEA recordings are becoming more commonly used there is no generally agreed-upon best method for sorting spikes (Swindale et al., 2017).

Approximately 90% of electrodes did not show any significant signs of recorded extracellular activity. This did not provide a good basis for a quantitative analysis on how gold and silver nanoparticles affect neuronal function. Using a different type of differentiation medium could increase the number of recording electrodes. Neurobasal-A (NBA) medium supplemented with 10% KnockOut Serum Replacement (KSR) and 1% penicillin-streptomycin was used in this study according to instructions from the supplier. However, BrainPhys medium has been used in MEA recordings with iCell® Neurons (30% glutamatergic and 70% GABAergic neurons) and iCell® Glutaneurons (90% glutamatergic neurons and 10% GABAergic neurons) (Tukker et al., 2018) and could provide better results. BrainPhys medium is comprised of BrainPhys neuronal medium supplemented with 2% iCell Neuron supplement, 1% Nervous System supplement, 1% penicillin-streptomycin, 1% N2 supplement and 0.1% laminin (10 μg/ml).

Adding hiPSC-derived astrocytes to the culture could be another solution to the problem with only a few recording electrodes on each MEA. Co-culturing with hiPSC-derived astrocytes promotes the frequency of spontaneous activity and synchronized bursts in hiPSC-derived neurons (Ishii et al., 2017). The MEA recordings by Ishii *et al.* were made with a MED64 System (Alpha MED Scientific) with 64 recording electrodes. When adding astrocytes, a co-culture with iCell® Glutaneurons and iCell® Astrocytes seems to be the best option for increased spontaneous activity compared to other co-cultures with hiPSC-derived neurons and astrocytes (Tukker et al., 2018). The MEA recordings by Tukker *et al.* were made with multiwell-MEA plates (Axion Biosystems Inc.) with 768 recording electrodes and more synchronized spiking activity was observed in this study as well when adding astrocytes. Synchronized electrical activity is an important feature for neuronal networks since it has been suggested to be involved in cognitive processes such as memory, attention and consciousness (Ward, 2003). Adding astrocytes would, apart from increasing electrical activity and synchronizing spike bursts, also make the cell culture resemble the *in vivo* brain more closely.

The electrophysiological recordings of the neuronal cultures were made with electrodes with a diameter of 10 µm instead of 30 µm due to better signal-to-noise ratio. However, the MEA plates with 30 µm electrodes might be a better alternative for future experiments since a larger electrode diameter might increase the probability of recording neuronal activity. Even though some difficulties occurred when recording electrical activity, MEA recordings of hiPSC-derived neurons do show a lot of promise and may enable a new approach to efficient animal free neurotoxicity testing in the future. MEA plates are well suited for high-throughput toxicity screening and could turn out to be a valuable tool when investigating possible health effects of nanoparticles and other compounds.

Conclusions

To conclude, the iCell® Neurons develop into functional neuronal networks and attach to electrodes on the microelectrode plates. Significant multiunit extracellular activity can be recorded from the neuronal cells with the setup presented in this thesis. However, further studies are needed in order to investigate the effects of gold and silver nanoparticles on neuronal function. No significant electrical activity associated with action potential waveforms was recorded from the hNPCs and further experiments are needed in order to develop a functioning assay for neurotoxicity testing.

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Appendix A

Table 1. *Technical specifications of the different MEA plates used in the study. For the transparent MEA type the contact pads and tracks are made of indium tin oxide (ITO) instead of titanium nitride (TiN). All MEA types have an internal reference (iR) electrode and the isolator material is silicon nitride (SiN).*

MEA type	Electrode number	Electrode diameter	Electrode spacing	Electrode material	Contact pads and tracks	Electrode grid
60MEA200/10iR-Ti	60	10 µm	200 µm	TiN	TiN	8x8
60MEA200/30iR-Ti	60	30 µm	200 µm	TiN	TiN	8x8
60MEA200/10iR-ITO	60	10 µm	200 µm	TiN	ITO	8x8