1 Introduction

1.1 Strategies for Drug Discovery

Toxicology is a complex ensemble of biochemical circuitry. It is the science of adverse effects in living organisms that drug candidates or chemical compounds are assessed for in a sequence of immunological or pharmacological actions and interactions. The assessment of these interactions in *in vitro* testing is an emerging challenge for toxicology as a translational science that transfers knowledge from basic science into practical applications in order to safeguard human health as well as the environment (Gundert-Remy *et al.*, 2015). Traditional toxicity testing relies on animal models, transformed cell lines or primary human cells. Despite the fact that animal models are limited by ethical constraints, cost, time, and failure to predict species-specific toxicity, animal models are still widely used in the assessment of safety and efficiency of chemicals (Yu & Thomson, 2014), (Stokes, 2015). In addition, rising costs and declining efficiency of drug research and development have imposed challenges on the pharmaceutical industry (Scannell *et al.*, 2012), (Paul *et al.*, 2010). A common consensus has by now been reached among the scientific community that drug failures in the clinical phases are primarily due to the poor predictive power of existing preclinical models due to species-specific toxicology, and that there is a critical need to develop new testing environments (Bowes *et al.*, 2012), (Caponigro & Sellers, 2011).

While the use of animal models has been predicted to be used for years (Zhang *et al.*, 2016) (Andersen & Krewski, 2009), or even decades (Stokes, 2015), there also seem to be scientific developments that indicate that the use of animal models may largely end within the next decade (Rowan, 2015). Over the past decades, the need for pharmacological and toxicological *in vitro* testing has gradually increased with the intention to develop specified treatments and simultaneously restrict animal testing (Collins *et al.*, 2008), (Tralau & Luch, 2012). The integration of physiological and pathological conditions in the form of cellularized, micro-engineered constructs forming *in*

vitro models holds the potential to solve this unmet need (Esch *et al.*, 2015). Recent progress has led to the development of new micro-devices, also referred to as organ-on-chip devices, which aim to recapitulate the parts of the *in vivo* microenvironment of living organs (Yum *et al.*, 2014), (Huh *et al.*, 2011). In order to test molecular compounds for their toxicology and efficiency to act as a drug, a model system needs to be able to reliably mimic key organ features *in vitro* (Park *et al.*, 2015), (Hebeiss *et al.*, 2012), (Tralau *et al.*, 2015), (Tralau & Luch, 2012).

While culture techniques allow cells and tissues to survive outside the body, they lack the ability to generate native-like functional tissues. In order for cells to form functional tissues, they need cues that mimic the native environments *in vitro*, such as the transport of nutrients, biochemical factors, and oxygen to the cells as well as removal of metabolic waste products (Zhong, 2010), (Martin *et al.*, 2004). This requires a vasculature *in vitro*, while static cell culture techniques rely on diffusional transport, which only allows an efficient exchange in the most superficial layer of a tissue (Chen & Hu, 2006). Here, biomimetic organ-on-chip systems offer several unique traits compared to *in vivo* animal tests, such as reduced sample consumption, high throughput, high-speed analysis, and improved sensitivity (Nge *et al.*, 2013). They further hold the potential to narrow or even close the gap of the 30% false-positive outcomes during clinical studies, which currently used *in vitro* bioassays and animal toxicity studies yield (Hvastkovs & Rusling, 2016). In part, the lack of toxicity detection is due to the difference in responses of humans and animals to chemicals (Shanks *et al.*, 2009). Therefore, one major challenge is to develop high-throughput toxicity tests that enable a more accurately predicted toxicity in humans and simultaneously fully elucidate the complex chemical pathways that lead to toxicity.

Several biomimetic platforms have been developed over the past years: The liver has been of particular focus as it is the key organ for drug-mediated toxicity. Kidneys are the main route for drug excretion and thus the second most affected organ for drug toxicity. Additional models have included biomimetic models to elucidate cardiotoxicity on the heart, pulmonary toxicity on the lung, and pancreatic toxicity. Additional emphasis has been placed on the blood-brain barrier (BBB), neurotoxicity, immunotoxicity, as well as developmental toxicity (Tralau & Luch, 2012).

1.2 The Central Nervous System

The central nervous system (CNS) consists of two parts, the spinal cord and the brain. While the CNS is comprised of seven major structures (medulla oblongata, pons, cerebellum, midbrain, diencephalon, cerebrum, and spinal cord) (Kandel & Schwartz, 2013), activity based functions represent a cornerstone of modern brain disease. Our cognitive abilities are, for example, primarily located in the *cerebral cortex* – the furrowed grey matter covering the cerebral hemispheres that is often affected in Alzheimer's disease. In contrast to that the *substantia nigra*, or midbrain, plays an important role in reward or movement and is often affected in patients with Parkinson´s disease.

The brain is the most intricate and complex organ in terms of composition and diverse, often still unknown functions. Due to its complex and delicate nature, the engineering of brain tissues is a rather new development compared to micro engineered tissues of other organs (Wobma & Vunjak-Novakovic, 2016). Neuronal tissue engineering has been employed to target patients enabling regenerative medicine (Chen *et al.*, 2016). In this work, the focus is not to create tissues for implantation and functional reconstruction, but rather to elucidate the mechanisms of the diseased brain with the aim to develop novel and improved treatments for patients.

1.2.1 Neurons and Synapses

The human brain is comprised of approximately 100 billion neurons, each receiving and sending signals to its thousands of connections. The signal transmittance is enabled through 100 trillion synapses. The complexity of the brain results from the neuronal organization into anatomical circuits with precise functions. The nervous system can be defined as the organized assembly of nerve cells and non-nervous cells. Neurons or nerve cells are specialized in the generation, integration, and conduction of incoming signals and delivering them to other neurons or effectors, such as muscle cells (Kandel & Schwartz, 2013). Neurons represent the basic elements of the nervous system. Despite the fact that the brain contains many different types of neurons, they all share the same basic architecture. A neuron consists of four morphologically defined regions: the soma,

dendrites, an axon, and presynaptic axon terminals (Figure 1). The soma is the metabolic center of the cell containing the nucleus. Dendrites form branch-like structures form the nucleus and form the central apparatus for receiving incoming signals from other neurons. The axon stretches a long way from the cell body and conducts signals to other neurons. At the end of the axon, synapses form at the axon terminals. These form the connection to transmit the signal from the presynaptic neuron (the axon) to the postsynaptic cells (the dendrite) at the synaptic terminal (Kandel & Schwartz, 2013).

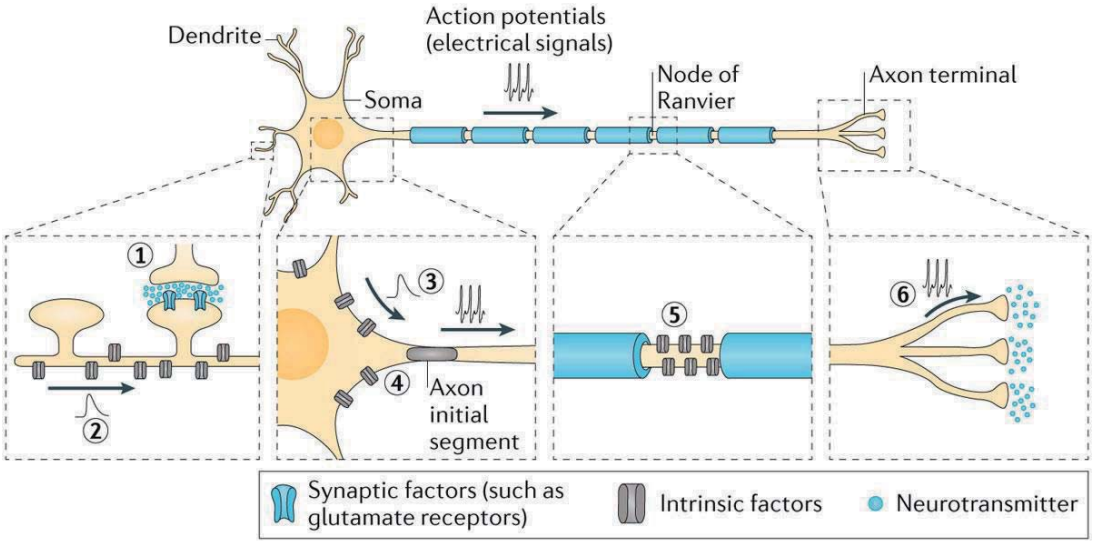

Figure 1 *Schematic drawing of synaptic and schematic excitability of a neuron. The presynaptic neurotransmitter release is a signal received by a neuron (1). A postsynaptic potential is generated and travels along the dendrite (2), the soma (3), the axon hillock and the axon initial segment (4). If the signal reaches a threshold, an action potential is generated and is influenced by intrinsic factors such as the nodes of Ranvier (5). At the axon terminal (6) the electrical signal triggers a neurotransmitter release. Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Neuroscience (Kourrich et al., 2015), copyright 2014 of Nature Publishing Group.*

Neurons maintain an electrical potential [V] across their membranes called the membrane potential, which is typically at around -65 mV ($V = V_{intracellular} - V_{extracellular}$). The membrane potential results from the unequal distribution of electrically charged ions, in particular Na^+ and K^+ ions, and the selective permeability of the membrane to only K^+. Na^+-K^+ pumps maintain the potential across the neuronal membrane (Kandel & Schwartz, 2013).

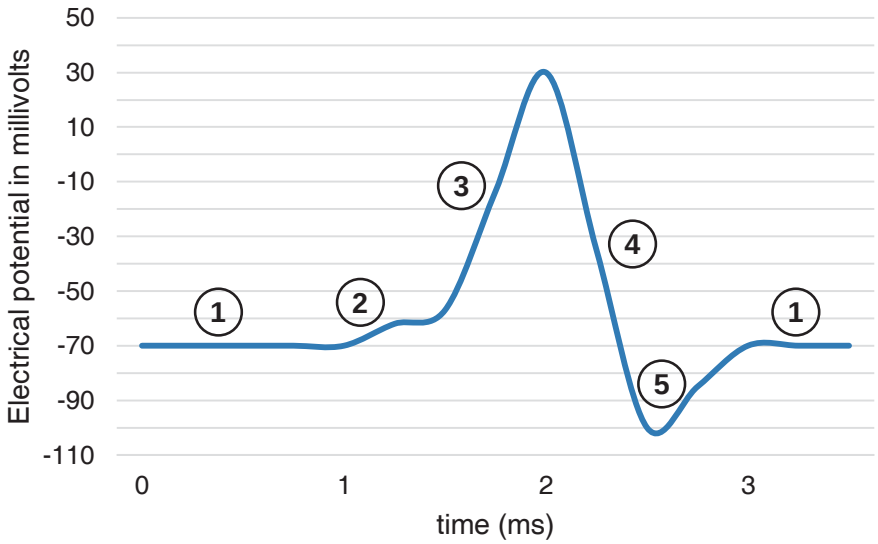

Figure 2 *Schematic drawing of an action potential.*
At the resting state sodium and potassium channels are closed and the membrane potential is maintained at -70 mV ***(1)****. A stimulus leads to sodium channel activation leading to membrane depolarization* ***(2)****. If a threshold is reached, an action potential is triggered leading to an opening of most sodium channels on the membrane leading to a positive membrane potential* ***(3)****. During the depolarization phase sodium channels close, while most potassium channels open thereby leading to a negative membrane potential* ***(4)****. The potassium channels lead to a hyperpolarization of the membrane potential* ***(5)*** *upon which sodium channels restore the membranes resting potential* ***(1)****.*

At the synaptic terminals, an excitation can occur when a neurons membrane potential can be altered quickly and significantly. In order to transmit a signal, the presynaptic neuron needs to receive an input (synaptic or sensory signal mediated by receptors). This in turn generates an influx of Na^+ or Ca^{2+} ions that leads to a depolarization of the membrane. If the depolarization exceeds a threshold, the Na^+-gated ion channels open thereby leading to a sudden increase of the electrical potential [V]. Upon closing of the Na^+-gated ion channels, the voltage-gated K^+-channel open, leading to an outflow of K^+ ions thus reestablishing the negative membrane potential. This process is outlined schematically in Figure 2 (Kandel & Schwartz, 2013).

An action potential functions with an all-or-nothing concept. If the stimulus lies below a certain threshold, no signal will be propagated. All stimuli that lie above the threshold

produce the same signal as outlined in Figure 2 and are propagated to post-synaptic neurons in the network (Kandel & Schwartz, 2013).

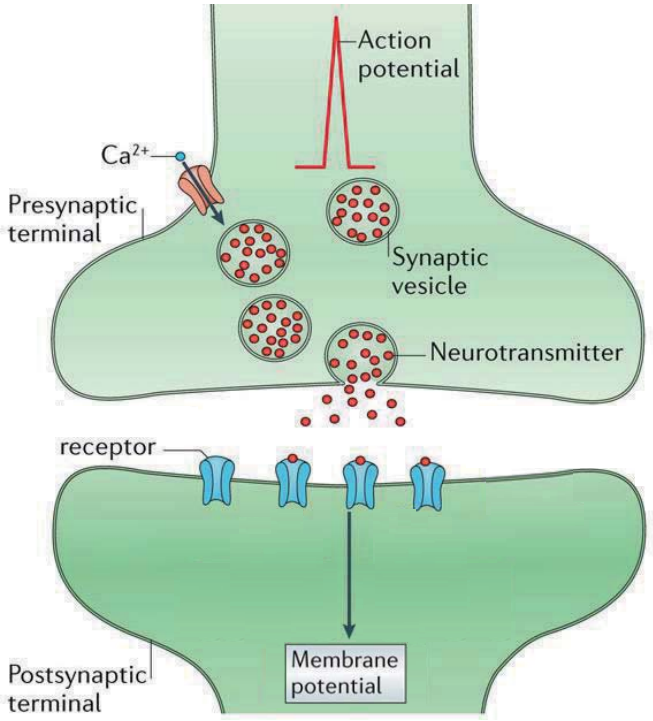

Figure 3 *Schematic drawing of a synapse and the synaptic terminal. Action potentials arriving at the presynaptic terminal lead to an opening of voltage-gated calcium channels. The increase of calcium concentration causes neurotransmitter containing synaptic vesicles to fuse with the presynaptic cell membrane, releasing neurotransmitter molecules into the synaptic cleft. The neurotransmitter molecules diffuse across the synaptic cleft and bind receptors on the postsynaptic membrane. The receptors in turn open (or close) ion channels, thereby changing the membrane potential of the postsynaptic cell. Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Neuroscience (Pereda, 2014), copyright 2014 of Nature Publishing Group.*

When an action potential reaches the pre-synaptic terminal of a neuron, it initiates an opening of voltage-gated calcium channels. The intracellular calcium increase leads to fusing of the vesicles with the presynaptic cell membrane, thereby releasing the chemical neurotransmitter (see Figure 3). The neurotransmitters diffuse across the synaptic cleft, a small space between the pre-synaptic and post-synaptic neuron, and bind post-synaptic receptors on the post-synaptic neurons. Binding of the neurotransmitters to the receptors causes a stimulus in the post-synaptic cell, generating an action potential if a threshold is reached (Kandel & Schwartz, 2013).

In the CNS, the main excitatory neuro-transmitter molecule is L-glutamate; its actions are counterbalanced by γ-aminobutyric acid (GABA) (Kandel & Schwartz, 2013). GABA is the main inhibitory transmitter in the brain (Reis *et al.*, 2009).

1.2.2 Alzheimer's disease – an example of neurological disorders

Defects in neuronal circuits may lead to the development of neurological disorders. Neurological disorders mostly develop out of sight and the complexity and inaccessibility as an organ makes the brain the most difficult organ to study.

Alzheimer's disease (AD) as an example of a neurodegenerative disorder is the most common form of dementia among elderly humans (Wilson *et al.*, 2012), (Alzheimer's, 2010) and primarily results from the degeneration or death of nerve cells in the cerebral cortex and other areas that usually control functions such as memory, personality, or logical thinking (Kandel & Schwartz, 2013). It affects approximately one-eighth of people older than 65 years. Demographic trends point towards an ageing population, which in combination with a higher risk for AD at higher age is likely to lead to larger absolute numbers of AD incidences. Experts expect the number of people affected by AD to triple over the next 25 years. Under these circumstances, AD is one of society´s major public health problems in western countries (Kandel & Schwartz, 2013).

Alois Alzheimer first described the clinical features and pathological changes of AD in 1906 as a progressive neurodegenerative disease (Alzheimer's, 2016). However, only in the 70s AD became a significant area of research, when it was recognized as the most common cause of dementia and a major cause of death (Katzman, 1976).

Recent research indicates that physiological changes of Alzheimer´s disease may already begin 20 years or more before symptoms appear (Villemagne *et al.*, 2013), (Reiman *et al.*, 2012), (Bateman *et al.*, 2012), (Jack *et al.*, 2009). That is why researchers believe that the early detection of Alzheimer´s will be key to preventing, slowing, and stopping the disease. This also led to recent progress in establishing new diagnostic criteria and guidelines for Alzheimer´s disease (Sperling *et al.*, 2011), (Albert *et al.*, 2011), (McKhann *et al.*, 2011).

These state that brain changes of Alzheimer´s begin before symptoms are detected, while earlier criteria required symptoms for a diagnosis to be made (McKhann *et al.*, 1984).

To date, the causes of AD are not yet known, although it is believed that multiple factors lead to its onset, as opposed to a single cause.

Examples are the β-amyloid peptide (Aβ) and the Tau protein (Maccioni *et al.*, 2001). Pathological symptoms are the formation of senile plaques in the brain, which are formed by a deposition of Aβ fibrils. Aβ is a fragment derived from the processing of amyloid precursor protein (APP). Plaques containing an aggregated form of Aβ fragments however, do not reliably indicate the presence of AD, as they have also been confirmed in healthy elderly subjects, although they do seem to contribute to the mechanism underlying the physiopathology of AD. APP is a transmembrane glycoprotein that plays a complex role in the cell with several attributed functions, such as serine protease inhibition, involvement of cellular adhesion and neuroprotection (Neve *et al.*, 2000), (Zheng *et al.*, 1998), (Kitaguchi *et al.*, 1988). In addition, the Tau protein, a multifunctional microtubule-associated protein, is believed to play an important role in AD. Tau is a major component of paired helical filaments (PHFs) and plays an important role in the assembly and stabilization of microtubules (Saragoni *et al.*, 2000), (Maccioni & Cambiazo, 1995), (Mandelkow *et al.*, 1995), (Kosik *et al.*, 1986).

Of AD diseased patients, only a fraction of less than 1% of cases is believed to be caused by a genetic mutation. In addition, no treatment to slow or stop the deterioration of brain cells in AD is available; although few drugs are approved that temporarily slow the worsening of symptoms (Alzheimer's, 2016). Between 2002 and 2012, 244 drugs for Alzheimer´s were tested in clinical trials worldwide (Cummings *et al.*, 2014). Only one of these clinically drugs received FDA approval in the U.S., making it a total of six drugs available to treat Alzheimer’s temporarily with symptomatic cognitive-enhancers or disease-modifying agents (Alzheimer's, 2016).

Developing effective treatment is difficult. Factors include the high cost of drug development and the long time required to observe whether a treatment affects disease progression. Further the brain is the most inaccessible organ of the body protected by the

blood-brain barrier, that only few and specialized small-molecule drugs can cross (Alzheimer's, 2016).

In comparison to current drug testing methods, neuronal tissue systems can therefore complement *in vivo* studies in animals and humans and serve as a controllable model for drug testing as well as disease modeling (Yi *et al.*, 2015b), (Booth & Kim, 2012). There is a growing need for *in vitro* disease models to better understand the etiology and enable faster development of treatment strategies.

1.3 Organ mimicking systems

The development of *in vitro* tissues and the mimicry of organ-like functions entails a tightly regulated mechanism of biological cues and external structural or physical stimuli.

1.3.1 *In vitro* Neuronal Networks

Recently, much effort has been put into creating simplified, high-throughput *in vitro* CNS models (Yi *et al.*, 2015a). Various *in vitro* disease models have been developed, such as for Alzheimer´s Disease or Parkinson´s Disease. They include techniques that enable the manipulation of axons, dendrites, synapses and neuronal networks. Systems with different culture conditions have been developed such as compartmental cultures, co-cultures, or 3D cultures (Yi *et al.*, 2015a). These simplified and high-throughput models are able to mimic *in vivo* situations by mimicking the 3D structure through incorporation of vasculature and the blood-brain barrier (van der Meer & van den Berg, 2012). These CNS models can be distinguished into axons, co-cultures of neuronal cells, neuronal networks with directionality, brain slices, and the read out of neuronal activity via microelectrode arrays (MEAs) (Lu *et al.*, 2012b), (Majumdar *et al.*, 2011), (Berdichevsky *et al.*, 2010), (Kunze *et al.*, 2011), (Park *et al.*, 2006), (Musick *et al.*, 2009).

1.3.2 2D vs. 3D and Encapsulation

Until today, most *in vitro* systems that serve to study neurological diseases have been based on two-dimensional (2D) culture methods (Park *et al.*, 2015). However, these cultures possess limitations in creating more complex cell-cell interactions and do not exhibit the natural morphology. The natural environment for cells is a complex and dynamic extracellular matrix where cells are able to react with countless biochemical and biophysical cues that direct function, regulate tissue homeostasis or pathophysiological events. Therefore, three-dimensional (3D) cultures represent the natural microenvironment much more accurately and allows to build functional cellular platforms to study, for instance, diseased tissues while simultaneously reducing the sacrifice of mice for biomedical studies (Kandel & Schwartz, 2013).

3D engineered tissues are able to mimic the *in vivo* structure, function, and response to drugs of human tissue more accurately than 2D models, which therefore leads to better *in vitro* models (Wobma & Vunjak-Novakovic, 2016), (Griffith & Naughton, 2002). In three-dimensional (3D) cultures, cell-cell contacts and interactions are important for morphology as well as cell signaling. Several studies have recently demonstrated differences between 2D and 3D cultures, specifically emphasizing the importance of 3D culture effects on neuronal cell phenotypes (Baker & Chen, 2012), (Frampton *et al.*, 2011), (Cullen *et al.*, 2011), (Irons *et al.*, 2008), (Smalley *et al.*, 2006), (Schindler *et al.*, 2006).

The encapsulation of cells in 3D structures to create engineered tissues is a relatively new research area, where 3D scaffolds act as a microenvironment for encapsulated cells (Utech & Boccaccini, 2016). In contrast to that, in conventional cell culture methods, cells are seeded on top of a surface. Cell interaction with surfaces in 2D space is an unnatural interaction. Even if the surface structure may be bent, the cells still adhere to the surface and are not immersed in it, which may ultimately affect a cell´s fate (Gieni & Hendzel, 2008), since *in vivo* cell-cell and cell-matrix interactions occur in a 3D space (Ferris *et al.*, 2013), (Hurtley, 2009), (Lee *et al.*, 2008). In order to create 3D *in vitro* biomimetic systems it is therefore paramount to enable an embedding into a 3D porous substrate. The extracellular matrix (ECM), which is the natural cellular environment *in vivo*, is a fibrous