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Establishing lateral entorhinal cortex layer II-neuron cultures as a tool for the investigation of early Alzheimer's disease related changes

Master's thesis in Neuroscience
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ABSTRACT

Alzheimer's disease is a slowly progressing and ultimately fatal neurodegenerative disease affecting more than 46 million people worldwide in 2018. The entorhinal cortex (EC), in particular the layer II of the domains located towards the collateral/rhinal fissure, contains neurons that are among the very first to undergo pathological alterations associated with the disease. Specifically, neurons in this domain develop the initial cortical tau pathology, while layer II is also known to exhibit severe neuronal loss already during the pre-clinical stages of AD. Furthermore, layer II-neurons are also subject to early accumulation of intracellular Aβ. In order to help enable the study of these early neuronal pathologies the work in this thesis was aimed at developing a platform for studying the LEC layer II neuronal population *in vitro.* Here, we have developed a protocol for the surgical extraction and culturing of adult LEC layer II-neurons from an AD-phenotype mouse model. Fourteen different experiments were performed to optimize the protocol being able to achieve high neuronal viability. My results demonstrate that it is possible to 1) identify LEC layer II-neurons on fresh vibratome sections, 2) selectively dissect these out while maintaining tissue integrity, and 3) dissociate and culture such neurons to the point that they re-form structural connections. Neuronal attachment was obtained up to two weeks after plating of neurons, and increasing complexity of the LEC layer II-neurons *in vitro* was observed, where the development of structural connections was achieved. In conclusion, culturing of LEC layer II-neurons is possible, though, future work should focus on optimizing the *in vitro* protocol to increase the viability of neurons and enable the measurement of neuronal activity.

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ABBREVIATIONS

AD	Alzheimer's disease
APP	Amyloid precursor protein
APOE	Apolipoprotein E
AOA	Age of animals
B-27	B-27 Plus Supplement
CA1	Cornu Ammonis 1, hippocampal subregion
CA3	Cornu Ammonis 3, hippocampal subregion
EC	Entorhinal cortex
fAD	Familial Alzheimer's disease
FBS	Fetal Bovine Serum
HF	Hippocampal formation
INS	Initial neuronal survival
LEC	Lateral entorhinal cortex
MEAs	Microelectrode arrays
MEC	Medial entorhinal cortex
MTL	Medial temporal lobe
NCM	Neuronal Culture Medium
NPM	Neurobasal Plus Medium
NFTs	Neurofibrillary tangles
PDL	Poly-D-Lysine
PLO	Poly-L-Ornithine
PHR	Parahippocampal region
PS1/PS2	Presenilin 1 and 2
PS	Penicillin-streptomycin
RI	Rock inhibitor
sAD	Sporadic Alzheimer's disease

1.0 INTRODUCTION

1.1 ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is a slowly progressing and ultimately fatal neurodegenerative disease affecting memory and cognitive functions. The disease is named after the neuropathologist Alois Alzheimer, who in 1901 observed symptoms like impaired memory, disorientation, aphasia, auditory hallucinations and increased confusion when examining his patient Auguste D. (Mauerer, Volk & Gerbaldo, 1997; Hippius & Neundörfer, 2003). After the death of Auguste D., Alois performed autopsy on her brain, where he described distinctive changes in the brain, including the formation of neurofibrillary tangles inside neurons, deposition of amyloid plaques in the extracellular space and atherosclerotic changes (Mauerer et al., 1997; Hardy, 2006; Hippius & Neundörfer, 2003). These findings have become well known as the pathological hallmarks of AD. More than a century later after Alzheimer's findings, we now have a greater understanding of the disease, both in terms of scope and severity.

Dementia is the fifth leading global cause of death (World Health Organization, 2018), where AD is the most common cause of dementia accountable for 60-80% of all cases (Prince et al., 2013). In 2018, there were more than 50 million people affected by dementia, and this number is expected to double every 20 years (Patterson, 2018). Estimates made in 2015 suggested that 131 million subjects would be affected by a dementing disease by 2050; however, updated estimates from 2018 increased this to 152 million, a prognostic increment of 14% in only four years (Prince et al., 2015; Patterson, 2018). The global economic costs of dementia reached 1 trillion US dollars in 2018 and are further expected to rise to 2 trillion US dollars by 2030 (Patterson, 2018). Every three seconds a new case of dementia arises (Prince et al., 2015), and we do not have any reason to believe it is going to decrease unless interventions delaying or preventing the onset of disease are achieved through medical and scientific breakthroughs. Since the beginning of AD drug development over 30 years ago, only four out of one hundred drugs have been approved. Moreover, these four drugs only partly treat symptoms (Patterson, 2018). The dramatic increase of dementia worldwide, coupled with the absence of effective treatment, and rising social costs, compels us to find a solution through research in the pre-clinical stage of AD to prevent or cure the disease.

1.2 AD RISK FACTORS

1.2.1 SPORADIC AD

Age is the main risk factor for developing AD, with the risk doubling every 5-6 years from the age of 65. (Ziegler-Graham et al., 2008). In sporadic AD (sAD), disease onset is typically after the age of 65 and accounts for 95% of all subjects affected (Piaceri, Nacmias & Sorbi, 2013). The second major risk factor for developing AD is determined by whether a subject is carrying the ε4 allele of the *ApoE-gene* (Reitz & Mayeux, 2014). The Apolipoprotein E (APOE) is a lipid binding protein and is expressed from the *APOE*-gene on chromosome 19. APOE is expressed via three major alleles, APOEε2, ε3, or ε4, and everyone will carry a combination of either of them (Reitz & Mayeux, 2014). The most common variant is APOEε3. Compared with this common variant, APOEε4 is associated with an increased risk for development of sAD. Specifically, if a subject carries one copy of APOEε4, the lifetime risk for AD is two to three-fold higher. With two copies of APOEε4, the lifetime risk is up to five-fold or even higher compared to the risk for those having two copies of APOEε3 (Reitz & Mayeux, 2014). It is relevant to point out though, that even if a subject carries two copies of APOEε4, AD development is not a given. Meanwhile, APOEε2 seems to have a protective effect against AD (Corbo & Scacchi, 1999). As about 40% of all cases in sAD are associated with APOEε4, the remaining 60% are considered to relate to other genetic and environmental factors (Piaceri et al., 2013).

1.2.2 FAMILIAL AD

Familial AD (fAD) is a far rarer form compared to sAD, where disease onset of fAD is typically before the age of 65 and accounts for 1-5% of all AD cases (Reitz & Mayeux, 2014). Disease development in fAD is due to a mutation in either of three genes; the *amyloid precursor protein*-gene (*APP*-gene) located on chromosome 21, the *Presenilin 1*-gene (*PSEN1*-gene) located on chromosome 14, or the *Presenilin 2*-gene (*PSEN2*-gene) located on chromosome 1 (Campion et al., 1999; Reitz & Mayeux, 2014). Mutations in the *APP-gene* are responsible for 9% of all fAD cases. These mutations are associated with increased accumulation of neurotoxic amyloid beta 42 fragments (Aβ42), resulting from increased APP cleavage by way of the amyloidogenic pathway (Piaceri et al., 2013; see section 1.3 for further description). Mutations in the *PSEN1*- and *PSEN2-genes* account for the majority of fAD cases. Both these genes encode major components of γ-secretase, a protease involved in proteolytic cleavage of APP resulting in the production of Aβ, in

particular, more of the Aβ42 form. Subjects carrying a mutation in the *APP*-gene or the *PSEN1*-gene are generally affected at younger ages, with disease onset at 45-65 years and 35-45 years, respectively, compared to those having fAD mutations in the *PSEN2*-gene that may develop the disease anywhere between 40-85 years (Barber, 2012).

1.3 AD PATHOLOGY

Along with severe, region-specific neuronal loss, the presence of large numbers of amyloid plaques (Thal et al., 2002) and neurofibrillary tangles (NFTs; Braak & Braak, 1991) represent the hallmarks of AD pathology. Plaques and NFTs are related to the accumulation of Aβ-fragments in the brain parenchyma and to intracellular cytoskeletal changes arising from the hyperphosphorylation of the microtubule-associated protein (MAP) tau in neurons, respectively (Fig. 1).

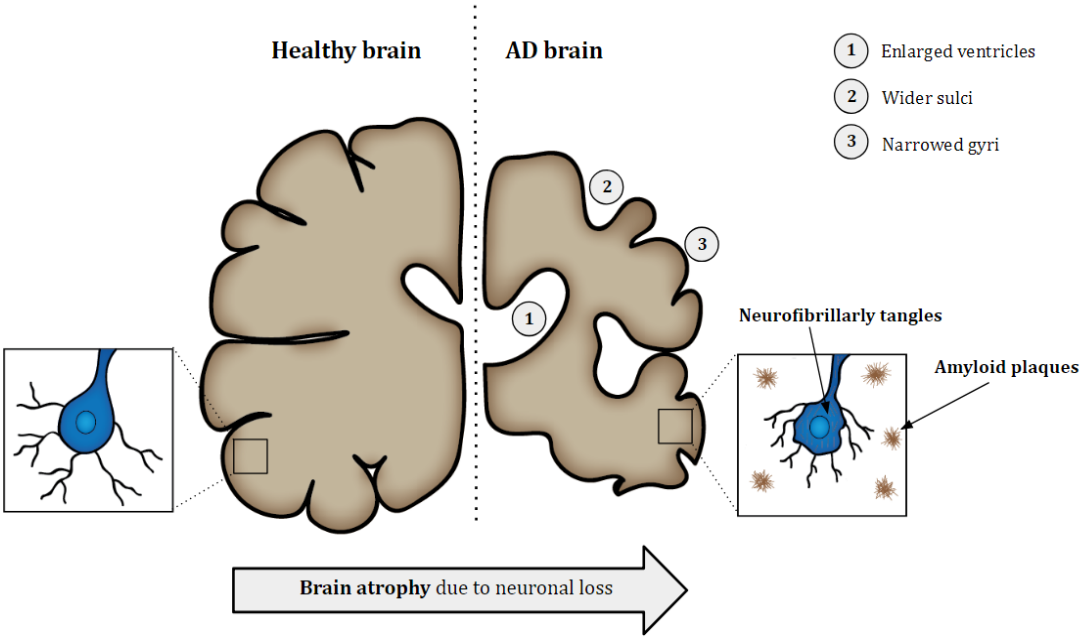

FIGURE 1. HEALTHY BRAIN COMPARED TO BRAIN AFFECTED BY ALZHEIMER'S DISEASE (AD).

In the AD brain, amyloid plaques and neurofibrillary tangles are two of the main neuropathological hallmarks (Thal et al., 2002; Braak & Braak, 1991). During the course of the disease, gross brain atrophy results from a massive neuronal loss (Gòmez-Isla et al., 1996), easily observed in the late stage AD brain as widening of sulci, narrowed gyri and enlarged ventricles (Liu et al., 2012) Figure modified from (University of Rochester Medical Center, 2019).

Aβ-peptides arise because of cleavage of APP by sequential proteolytic processing in what is known as the amyloidogenic pathway (Fig. 2; Zheng & Koo, 2006). In this pathway, the APP N-

terminal is first cleaved by β-secretase (BACE-1), releasing a secreted APP ectodomain (sAPPβ). The remaining 99 amino acid C-terminal (C99) is further cleaved by γ-secretase into the APP intracellular domain (AICD) and the Aβ-peptide. Aβ-peptides can vary in length from 38-43 amino acid residues, where Aβ42 constitutes the most toxic form, being more prone to form oligomers, fibrils and amyloid plaques (Fig. 3). In the non-amyloidogenic pathway, the N-terminal of APP is proteolytically cleaved by α-secretase to release a secreted APP ectodomain (sAPPα). The remaining C-terminal (C83) is subsequently cleaved by γ-secretase forming a small peptide called p3 along with an AICD. These Aβ-peptides will accumulate, subsequently forming sticky insoluble Aβ-plaques.

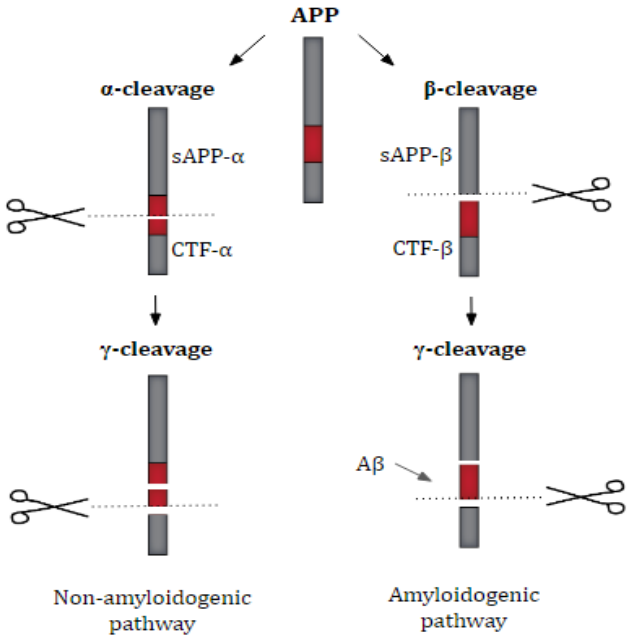

FIGURE 2. AMYLOIDOGENIC- AND NON-AMYLOIDOGENIC PATHWAY.

The amyloid precursor protein (APP) will be enzymatically cleaved into smaller fragments by either of two proteolytic pathways (Zheng & Koo, 2006). The amyloidogenic pathway consists of initial cleavage by β-secretase, resulting in secreted APP-β and C-terminal β. The latter is subsequently cleaved by γ-secretase resulting in the production of Aβ. The non-amyloidogenic pathway consists of initial cleavage by α-secretase, resulting in secreted APP-α and C-terminal α. This processing precludes the possibility for cleaving out Aβ, and rather, the subsequent cleavage of the C-terminal fragment by γ-secretase results in a small fragment called p3. Meanwhile, both pathways leave the intracellular domain of APP (Zheng & Koo, 2006).

Aβ-plaques can be differentiated as dense-core or diffuse plaques present in the extracellular space (Rak et al., 2007). Dense-core plaques contain neuritic dystrophies and are correlated with synaptic loss and activated microglia. This type of plaques is also linked to cognitive impairment. Contrary, diffuse plaques are rather amorphous deposits that lack a dense core as well as dystrophic neurites, and these plaques do not seem to impair cognitive functions (Rak et al., 2007). Amyloid plaques can disrupt synaptic signaling and trigger an immune response, activating inflammatory processes causing damage to surrounding neurons (Shemer et al., 2006). Neuroinflammation can in return aggregate the phosphorylation of tau and increase the amyloid plaque burden (Kinney et al., 2018).

In the neuron cytoskeleton, the tau protein is crucial for the stability of microtubules which enable substances required for normal cell-functioning to be transported along dendrites and axons (Braak & Braak, 1998). In AD, these tau proteins become hyperphosphorylated, thus causing microtubules to dissociate and become unstable, subsequently disrupting normal cell functioning. (Simic et al., 2016). The defective tau protein consequently creates filaments, where small dimers eventually aggregate into oligomers (Fig. 3). In the oligomeric stage, these abnormal tau depositions form paired helical filaments (PHF) before aggregating into insoluble NFTs. When these defective tau proteins are in the oligomeric state, they are neurotoxic and cause synaptic dysfunction and impairment of long-term potentiation, which is crucial for memory formation (Simic et al., 2016).

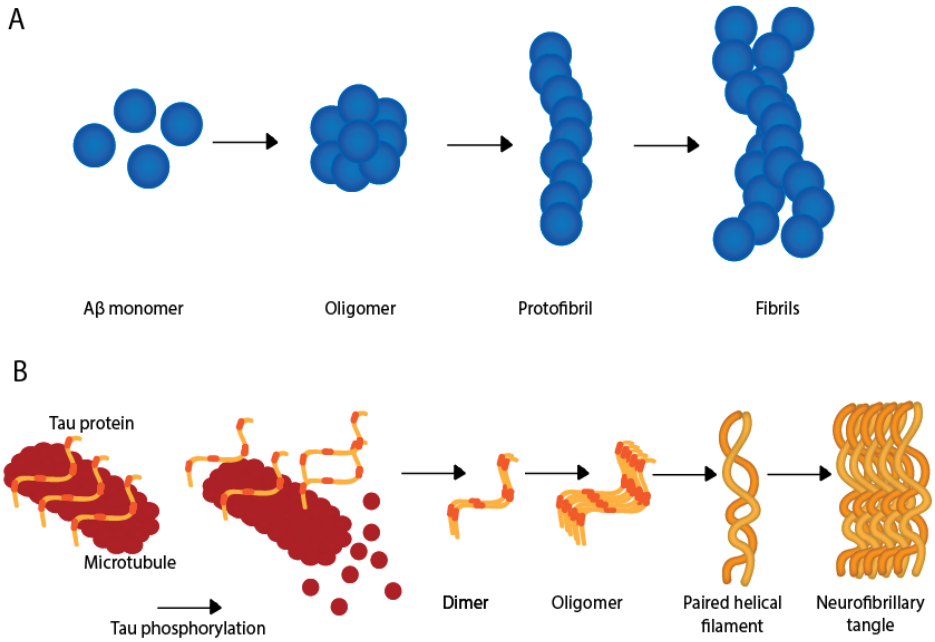

FIGURE 3. AGGREGATION OF AB AND TAU IN ALZHEIMER'S DISEASE.

(A) Aβ monomers, more in particular of the -42 form, tend to be sticky, and will tend to aggregate and form amyloid plaques by a sequence of aggregating steps involving oligomers, then protofibrils and in the end fibrils. **(B)** When tau proteins which in a healthy state stabilize microtubules gets hyperphosphorylated, they detach from microtubules making these unstable. Tau will then tend to aggregate into oligomers, then form paired helical filaments, and eventually form neurofibrillary tangles. Figure adapted from (Salahuddin et al., 2016 (A); Šimic et al., 2016 (B)).

1.4 BRAIN AREAS INVOLVED EARLY IN AD

1.4.1 ANATOMICAL STAGING OF AB-PLAQUES AND NFTS

The evolution of Aβ-plaques can be divided into five phases (Thal, et al., 2002). In the first phase Aβ-plaques are located exclusively in the neocortex (Thal et al., 2002). Secondly, allocortical brain regions become involved, including entorhinal cortex, CA1 and insular cortex. In the third phase Aβ-plaques are seen in diencephalic nuclei, striatum and cholinergic nuclei of the basal forebrain, subsequently spreading to several brain stem nuclei in phase four. In the last phase, cerebellar Aβ-plaques are included. Meanwhile, NFTs initially form in layer II of the entorhinal cortex (EC), before spreading to the hippocampal formation (Braak & Braak, 1991). As the disease progresses temporal, frontal and parietal neocortex are also affected by NFTs, and in the end stage of AD, unimodal, primary sensory and motor areas of the neocortex become affected (Braak & Braak,

1991). The total amount of Aβ load seems to peek in the stage of the disease when clinical symptoms appear, or even at preclinical stages, while the proportion of NFTs, on the other hand, seems to increase throughout the clinical course of the disease (Serrano-Pozo et al., 2011).

1.4.2 BRAIN ATROPHY

The hippocampal and parahippocampal structures located in the medial temporal lobe (MTL) show atrophy at early stages of AD (Dickerson & Sperling, 2008). The EC is considered one of, if not *the* first area affected by neuronal loss. More specific, laminar differences are apparent where 60% neuronal loss is observed in EC layer II in patients with very mild AD compared with controls, while dramatically ~90% neuronal loss is observed in patients with severe AD (Gomez-Isla et al., 1996). By late stages of the disease, when several brain areas are heavily affected, major atrophy of gyri and widening of sulci is plainly evident, no doubt due to massive neuronal loss (Van Hoesen, Hyman & Damasio, 1991).

1.5 EC ANATOMY

The EC contains six layered periarchicortex where layer II-, III-, V-, and VI are cellular, while layer I and layer IV (lamina dissecans) are largely acellular (Cappaert, Van Strien & Witter, 2015). While several subfields are recognized in EC, it is most commonly divided into two main subregions; the lateral entorhinal cortex (LEC) and the medial entorhinal cortex (MEC; Van Strien, Cappaert & Witter, 2009). In rodents, these two subdivisions may be further subdivided, with LEC consisting of a dorsolateral (DLE), dorsal intermediate (DIE), and ventral intermediate (VIE) subfield, and MEC consisting of a medial entorhinal (ME) and central (CE) subfield. In the rodent brain, EC occupies the ventrocaudal part, partially enclosed to the rhinal fissure dorsolaterally (as reviewed by Van Strien et al., 2009). Within EC, LEC occupies a more rostrolateral position, while MEC is positioned caudomedially (Canto, Wouterlood & Witter, 2008). In relation to other cortical areas, the anterior part of EC borders olfactory and amygdaloid cortices, where piriform cortex outlines the lateral part and the periamygdaloid cortical nucleus of the amygdala border parts of the ventromedial part (Canto et al., 2008). Medially EC further borders the parasubiculum and amygdala-hippocampal transition, while perirhinal cortex (PER), and postrhinal cortex (POR) border the lateral and posterolateral position, respectively.

The EC is an integral part of the hippocampal-parahippocampal circuitry (Fig. 4), where neocortical sensory information reaches the parahippocampal region (PHR), that further projects to the hippocampal formation (HF; Witter, 2010). The standard view (Fig. 4B) of the entorhinal-hippocampal network, consists of neocortical projections aiming at the PHR where two parallel projection streams from PER and POR, projects to LEC and MEC, respectively. Further, the EC layer II project to the dentate gyrus (DG) and CA3, while the EC layer III project to subiculum and CA1. DG projects to CA3 via the mossy fiber pathway, CA1 receive projections from CA3, and CA1 further projects to the subiculum. Subiculum and CA1 in turn projects to the deep layers V of EC, which in turn constitute reciprocal projections to PER and POR (Van Strien et al., 2015).

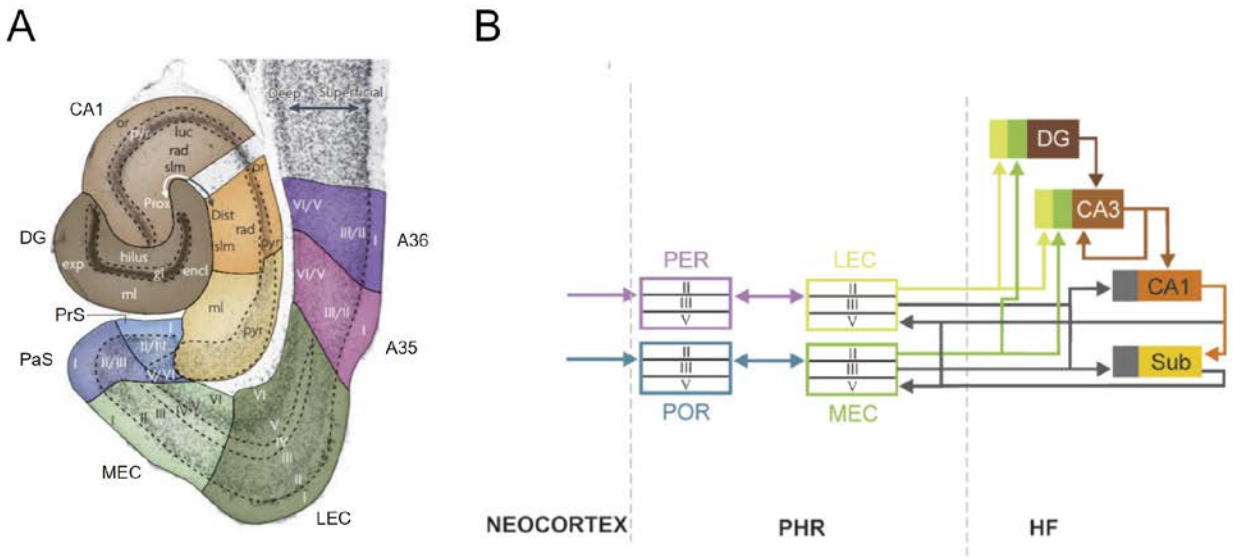

FIGURE 4. THE HIPPOCAMPAL FORMATION AND PARAHIPPOCAMPAL REGION.

(A) Figure show all areas involved in the hippocampal formation (HF) and parahippocampal region (PHR) from a Nissl-stained horizontal cross section. The HF consist of dentate gyrus (DG) marked in dark brown, CA3 in light brown and CA3 in orange, CA2 which is the uncolored area in between CA1 and CA3, and the subiculum marked in yellow. The PHR consist of presubiculum (PrS) marked in light blue, parasubiculum (PaS) in dark blue, medial entorhinal area (MEA) in light green, lateral entorhinal area (LEA), perirhinal cortex (A35) in pink and (A36) in purple. **(B)** The standard view of the entorhinal-hippocampal network. Neocortical projections reach the PHR, where two parallel projection streams are distinguished. PER projects to LEC, while POR projects to MEC. Further, layer II neurons of both LEC and MEC project to the DG and CA3, while layer III neurons project to CA1 and subiculum. These projections from EC layer II and III originate the perforant pathway reaching all sub-regions of the HF. Projections from all sub-regions of the HF reciprocate to layer V of EC, and EC in turn, send reciprocal projections to PER and POR from LEC and MEC, respectively. (Figure adapted with permission from Van Strien et al., 2009 (A) and Witter, 2010 (B)),

1.5.1 DELINEATING EC LAYER II

In terms of delineating LEC from MEC, laminar differences can be used. Layer II in both LEC and MEC contain large neurons, where in LEC these neurons show a characteristic clustering into 'islands' which is not prominent in MEC (Cappaert et al., 2015). Layer II neurons in LEC can further be subdivided into superficial layer IIa separated by a cell-free layer to the deeper layer IIb neurons (Cappaert et al., 2015). At the more dorsal portion of the brain layer II cells in LEC will extend into layer I compared to the neighbouring brain area, perirhinal cortex. The lamina dissecans (layer IV) is more prominent in MEC, where the acellular area is more apparent compared to a more widespread distinction in LEC. Also, a striking feature of layer V in MEC is the presence of radial arrangements of the pyramidal neurons, which is not seen in LEC. While these features is typically noted in basic dye preparations, they can also be noticed by a trained eye of fresh, unstained tissue, if appropriate contrast is achieved.

1.5.2 DELINEATING CA3 STRATUM PYRAMIDALE

Hippocampus consists of a three-layered archicortex, with a deep polymorphic layer (stratum oriens), middle principal layer and a superficial molecular layer (Burwell & Agster, 2008; Witter, 2010). The superficial layer is further subdivided into sublayers in the CA-fields; stratum lucidum, stratum radiatum, stratum pyramidale and stratum lacunosum moleculare (Burwell & Agster, 2008; Witter, 2010). As for the identification of CA3 pyramidal neurons, laminar differences can be identified and differentiated based on stratum pyramidale in CA3 cell bodies that are less densely packed than those in neighboring CA1. Proximal CA3 is located towards DG, while distal CA3 is towards CA1 (Burwell & Agster, 2008). In this thesis, the main bulk of CA3 was dissected out and cultured.

1.6 SIGNIFICANCE OF THIS THESIS

As we aim to better understand the initial changes related to Alzheimer's disease onset, we should aim to study the dysfunction of neurons that appear affected particularly early in the course of the disease. The EC layer II neurons are known to be selectively vulnerable, where massive neuronal loss is observed in pre-clinical stages (Gomez-Isla et al., 1996; Kordower et al., 2001). Particularly, based on fMRI measures of metabolic activity, LEC has been suggested to be affected initially (Khan et al., 2014), and this has been substantiated by volume measures (Olsen et al., 2017).

Furthermore, recent evidence suggests a link between reelin expressing neurons in EC layer II and the tendency to accumulate intracellular Aβ (Kobro-Flatmoen, Nagelhus & Witter, 2016). These reelin expressing neurons in EC layer II, consists mainly of fan cells in LEC and stellate cells in MEC (Witter et al., 2017), and seem to be associated with neurodegeneration in AD where reduction of reelin expression in EC layer II is found in both animal models and humans (as reviewed by Stranahan & Mattson, 2010). Further, fan cells in LEC layer II appear to be more altered compared to stellate cells in MEC, as judging by electrophysiological firing properties (Marcantoni et al., 2014). As reelin modulates synaptic plasticity, thus being important for memory and learning (Weeber et al., 2002; Chin et al., 2007) it is of particular interest in AD as alterations of reelin signaling can cause neuronal dysfunction (Xian et al., 2018). Furthermore, in individuals with early AD, it has been observed synaptic loss in the molecular layer of DG, one of the major projecting sites of LEC layer II neurons (Scheff et al., 2006). It would be highly interesting being able to study these demonstrated dysfunctions in LEC layer-II neurons at a single neuronal level to detect potential differences in neuronal morphology and excitability between AD-neurons vs controls. This could be done in a primary cell culture, where different parameters can be investigated at a single neuronal level.

1.6.1 PRIMARY NEURONAL CULTURES

To enable a primary cell culture there are four main stages to consider: 1) obtained tissue from an animal by dissection, 2) isolate chunks of tissue, 3) dissociated tissue into isolated neurons, and 4) culture neurons on a coating plate (Freshney, 2016, p. 207). As far as we know, culturing of LEC layer II neurons extracted from adult mice has never before been conducted, and as these neurons seem to be selectively vulnerable in AD, we aim to study this neuronal population *in vitro.* Brain tissue for primary cortical cell cultures is usually extracted from animals at the embryonic stage, as at this time point neurons are not as fragile compared to those in postnatal animals (Sciaretta & Minichiello, 2010). This is because neurons at this stage not yet have developed extensive axonal and dendritic extensions, thus the neuronal network is still not fully developed (Sciaretta & Minichiello, 2010). In mice, the extraction of cortical neurons is usually done between embryonic days eleven to seventeen. However, as neuronal alterations caused by AD pathology, in terms of accumulation of Aβ and hyperphosphorylation of tau proteins occurs at postnatal age, it is not possible to investigate these alterations at embryonic stages. Culturing of neurons from adult rodents tends to be difficult, however, primary cell cultures with tissue extracted from adult mice

have previously been conducted on primary cortical-, (Eide & Murray, 2005) and hippocampal neurons, where neuronal survival and attachment were achieved (Brewer, 1997).

In order to maintain stability in an *in vitro* primary cell culture it is vital to isolate neurons by dissociation without inducing to much oxidative stress, maintain optimal culture conditions in terms of ionic balance, obtain good growth conditions to achieve neuronal attachment, and ensure the absence of contamination in the culture (as reviewed by Lodish et al., 2000). Also, co-cultures with astrocytes can be used, as the astrocytes-derived extracellular matrix has been demonstrated to result in higher attachment of neurons (Noble, Fok-Sang & Cohen, 1984).

1.6.2 AIM

Based on the known vulnerability of LEC layer II neurons in the early stages of AD, this master's thesis aimed to develop a protocol for the selective culturing of fully developed LEC-layer II neurons. To achieve this, we have 1) micro-dissected out LEC layer II-neurons from acutely harvested brains of young adult AD-transgenic mice as well as wild type controls, 2) tested fourteen different protocols where these neurons have been cultured under varying conditions in order to develop an optimal protocol with respect to neuronal viability, and 3) tested protocol for conducting experiments fitted for future electrophysiological experiments using multielectrode arrays.

2.0 METHODS

2.1 ANIMALS

In total, 19 young adult mice (female n=9, male n=10) were used in this project, where all animals used were provided by the Comparative Medicine Core Facility (CoMed) at the Norwegian University of Science and Technology (NTNU). All experiments described in this project was performed at Kavli Institute of Systems Neuroscience, Centre for Neural Computation and at the Department of Neuromedicine and Human Movement Science. All breeding, housing and handling of animals were approved by the Norwegian Animal Research Authority in accordance to the Norwegian Animal Welfare Act (§§ 1-28) and the Norwegian Regulations of Animal Research (§§ 1-26). All animals were held in standard lab cages (up to 5 animals per cage), in temperatures of 22 ± 2°C, and kept at a light/dark cycle of 12:12 hours, with access to food and water *ad libitum*. All experiments were performed during the dark cycle.

The AD model mice were of the APP/PS1 model (Radde et al., 2006), with littermates negative for the APP/PS1 construct serving as controls. The APP/PS1 model carries the human Swedish (KM670/671NL) mutation in the *APP*-gene and human L166P mutation in the *PSEN1*-gene. These mutations, both controlled by the Thy1 promoter, lead to the development of an AD-like phenotype with respect to increased accumulation of Aβ, including intracellular Aβ, and learning impairments (Radde et al., 2006). The sex, age and genotype of animals used in each experiment can be seen in Table.1.

Table 1. Animals used in experiments according to sex, age and genotype.

Experiment	Sex	Age	Genotype
1	F	P36	AD
2	M	P43	AD
3*	M	P28	Control
4*	F	P29	AD
5*	M	P36	Control
6	M	P38	AD
7	F	P59	AD
8*	F	P65	AD
9	M	P54	Control
10*	F	P62	AD
11**	F	P75	AD
12**	F	P75	AD
13	M	P87	Control
14*	M	P88	Control

*****Two animals used per experiment. ** Same animal used in experiment 11 and 12.

2.2 EXTRACTING BRAIN TISSUE

The animals were deeply anesthetized in a chamber filled with isoflurane (Abbott Lab., Cat#05260-05), and then decapitated with sterilized surgical scissors (FST, Cat# 14001-13) followed by immediate submersion in ice cold Hanks' Balanced Salt Solution (HBSS; Thermo Fischer Scientific., Cat# 88284). Subsequently, the brains were extracted from the scull while still submerged in ice cold HBSS in a petri dish resting on a platform of ice. Fine scissors (FST 14568-09) and S&T forceps (FST., Cat# 00108-11) were used to cut and then break up the scull, and a spatula (RSG Solingen., Cat# 231-2262) was used to severe the cranial nerves and gently pick the brains out. The brains were then directly transferred to a container with ice cold HBSS, which was again placed on ice, and kept there until sectioning on a vibratome (see below). All equipment used had been autoclaved and/or sterilized with 70% ethanol.

Each brain was horizontally sectioned at 300 µm in a ventral-to-dorsal plane at a rostral-to-caudal direction on a Leica VT1000 S vibrating blade microtome (Leica Biosystems. Cat# 14047235613;

frequency of 5.3 Hz, speed of 8 mm/s), while immersed in ice cold HBSS (Fig. 5). Sections were then transferred to individual wells containing Hibernate™-A Medium (Thermo Fisher Scientific., Cat# A1247501), resting on a platform of ice, before being transferred to a stereoscope for microdissection of LEC-layer II.

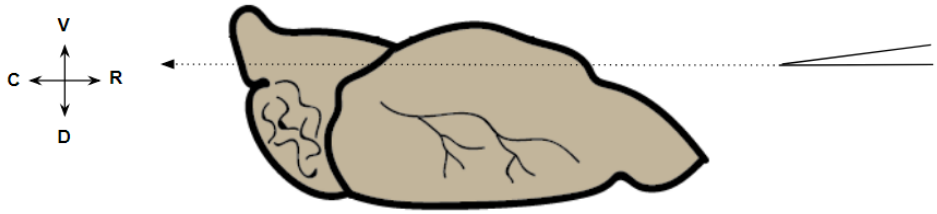

FIGURE 5. ILLUSTRATION OF HORIZONTAL SECTIONING OF THE MOUSE BRAIN.

Each mouse brain was cut in horizontal sections from the ventral-to-dorsal plane, and in a rostral-to-caudal direction. Abbreviations: D; dorsal, C; caudal, V; ventral, R, rostral.

For the microdissection, I used a custom-made dust reduction chamber build around a stereoscope (SteREO Discovery V8; Zeiss), which was cleaned by spraying it down with 70% ethanol between each use. Each individual section was transferred to an autoclaved petri dish placed upside-down on the stage of the stereoscope, using a plastic pipette that had been cleaned with 100% ethanol. The petri dish was soaked with Hibernate-A medium, and LEC-layer II was microdissected using surgical scissors (Vannas Spring Scissors (FST, Cat# 15000-08). Sections were continually soaked with Hibernate-A medium to prevent the tissue from drying out. After dissecting out each chunk of LEC-layer II tissue, it was immediately transferred to a Falcon Tube (15 mL) containing Hibernate-A medium embedded in ice.

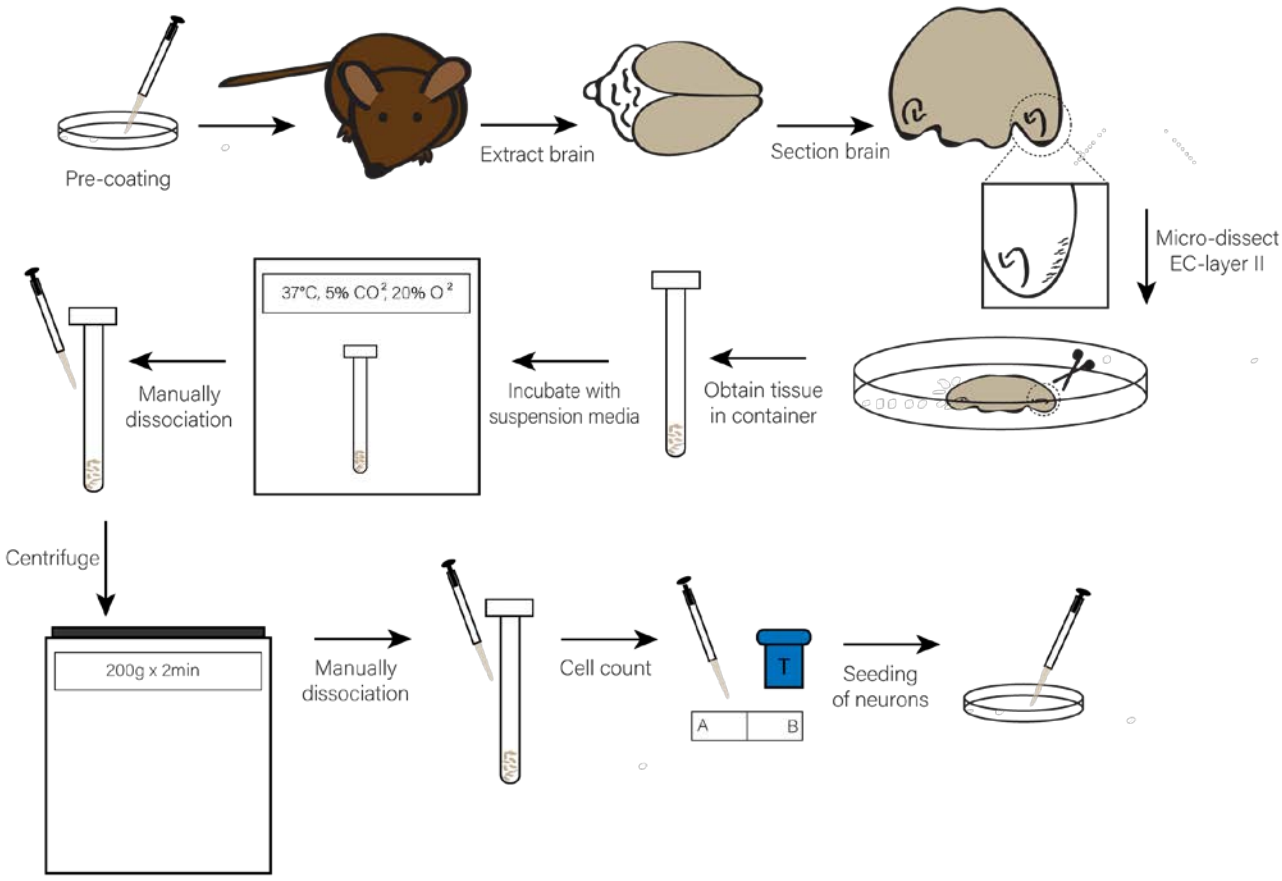

FIGURE 6. SIMPLIFIED ILLUSTRATION OF THE PROTOCOL PROCEDURE.

Coating of well-plates was performed 1-7 days before seeding of neurons. Brains were freshly extracted before being cut into horizontal sections on the vibratome. LEC layer II was then micro-dissected out under the stereoscope and held in a container with ice before being brought to a sterile lab. Tissue was then incubated in a humidified incubator (37°C, 5% CO^2, 0% O^2) with cell suspension containing cell media, protease and supplements, which was prepared 30 min to 5 hours ahead. Tissue was then manually dissociated by pipetting up and down ten times, followed by centrifugation at 200x g for 2 minutes. Dissociation was completed by manually pipetting up and down ten times once more. Before seeding neurons at pre-coated well-plates, cell counts were performed using an automated cell counter. (Figure modified from Carter & Shieh, 2015).

2.3 CULTURING OF EC LAYER II-NEURONS

2.3.1 NEURON CULTURE STEP BY STEP

An overview of the process regarding the culturing of freshly dissected neurons can be seen in Fig. 6. Dissected LEC-layer II tissue was immediately brought to a cell-culture lab (the lab of Dr. Ioanna Sandvig and Dr. Axel Sandvig) located on campus in about two minutes walking distance from the Kavli Institute. At the cell-culture lab, the media (Hibernate-A) were aspirated from the

tube containing the tissue before the tissue was transferred into a new sterile 1.5 mL tube with 1 mL media containing proteases, neuronal cell media and other supplements whose contents were systematically varied depending on which of the 14 protocols was being tested (see Appendix 1 for protocols; see Table 2). The tissue was then incubated in a standard humidified air incubator (37°C, 5% CO^2, 20% O^2) for 25 ± 5 minutes, before the medium was aspirated and ~100 µL of dissociation media were added. Dissociation of the tissue was furthered by triturating the tissue with a 100 or 1000 µL pipette, taking care not to blow air bubbles into the resulting cell suspension. The cell suspension was then transferred into a sterile 15 mL Eppendorf tube, fresh culture media up to 3 mL was added and cells were centrifuged at 200x g for 2 minutes. The supernatant was then aspirated, and the resulting cell pellet was re-suspended in 100 µL fresh cell medium. Dissociation of the tissue was completed by triturating with a 100 or 1000 µL pipette, avoiding blowing air bubbles into the cell suspension. Cell counts were then obtained using Trypan Blue Stain (Invitrogen™, Cat# T10282) in combination with cell suspension (50:50) and counted on a Countess II Automated cell counter (Thermo Fischer, Cat# AMQAX1000). Dissociated neurons were then divided into pre-coated wells (see appendix 1 for protocols), fresh cell medium was added, and the neurons were incubated in a standard humidified air incubator (37°C, 5% CO^2, 20% O^2) for a minimum of 24 hours before microscopic inspection. 1-5 days after plating the neurons, 50-80% of the total cell medium in the wells was exchanged with fresh medium.

In experiment 5, 11, 13 and 14, CA3 pyramidal neurons were dissected out and cultured along with LEC layer II neurons. Here, tissue was extracted using the same techniques and protocol as for LEC layer II neurons. These CA3 pyramidal neurons were included in the experiments to explore the possibility of future work *in vitro* with the aim of establishing contacts between LEC layer II and CA3 neurons. Astrocytes were seeded as a monolayer in experiment 3 and 11-13 (see appendix 2 for complete protocol). Exchange of the medium was repeated every three days until termination of the given culture. All media used during the protocol were prepared between thirty minutes and four hours before plating of the neurons, and this was always carried out using aseptic techniques in a laminar flow hood. Coating of the culture vessels was prepared 1-7 days before plating of neurons. Individual materials and chemicals used in protocols are listed in Table 3.

Table 2. Overview of media supplements, coating substrates and coating vessels in all 14 protocols.

Protocol	Cell suspension	Coating substrate	Coating vessels
1	NCM, dispase, FBS 1%, PS 1%	Corning matrigel	Ibidi® chip (8-well)
2	NCM, dispase, FBS 10%, PS 1%, L-glut 1%	Corning matrigel	Ibidi® chip (8-well)
3	NCM, dispase, FBS 10%, PS 1%, L-glut 1%, RI 1:1000	Corning matrigel Astrocytes	Ibidi® chip (8-well)
4	NCM, dispase, FBS 10%, PS 1%, L-glut 1%, RI 1:1000	PLO + Laminin Fibronectin	Ibidi® chip (8-well)
5	NCM, dispase, FBS 10%, PS 1%, L-glut 1%, RI 1:1000	PLO + Laminin	Ibidi® chip (8-well)
6	NPM w/GM+B-27, dispase, FBS 10%, RI 1:1000	PDL	Corning® 12 well-plate
7	NPM w/GM+B-27, dispase, FBS 10%, RI 1:1000, DNase I 1%	PDL	Corning® 12 well-plate
8	NPM w/GM+B-27, dispase, FBS 10%, RI 1:1000	PDL	Ibidi® chip (8-well)
9	NPM w/GM+B-27, dispase, FBS 10%, RI 1:1000	Geltrex + DMEM/F-12	Ibidi® chip (8-well)
10	NPM w/GM+B-27, dispase, FBS 10%, RI 1:1000, PS 0.5%	PDL	Corning® 96 well-plate
11	NPM w/GM+B-27, dispase, FBS 10%, RI 1:1000	PLO + Laminin + astrocytes	Microfluidic chips
12	NPM w/GM+B-27, dispase, FBS 10%, RI 1:1000	PDL + astrocytes	Corning® 96 well-plate
13	NPM w/GM+B-27, dispase, FBS 10%, RI 1:1000	PLO + Laminin + astrocytes	6 well MEA
14	NPM w/GM+B-27, dispase, FBS 10%, RI 1:1000	PDL	Corning® 12 well-plate

Overview of all media supplements, coating substrates and coating plates used in the 14 different protocols. Abbreviations: NCM; Neuronal culture medium, FBS; Fetal bovine serum, PS; Penicillin-streptomycin, L-glut; L-glutamine, RI; Rock inhibitor (Y-27632), NPM; Neurobasal plus medium, GM; GlutaMAX, B-27; B-27 Plus supplement, PLO; Poly-L-Ornithine, PDL; Poly-D-Lysine.

2.3.2 COATING OF CULTURE VESSELS

For the first five experiments along with experiment 8 and 9, 8 well chamber Ibidi chips (Ibidi® Cells in focus, Cat #80841) were used. These have a growth area of 0.93 cm^2 and a volume of 400 µL per well. For experiment 6, 7 and 14, Corning® 12 well plates (Sigma-Aldrich, Cat# CLS3512-100EA) were used. These have a growth area of 3.8 cm^2 and volume of 6.9 mL per well. In experiment 10 and 12 Corning® 96 well plates (Sigma-Aldrich, Cat# CLS9102) were used, and

these have a growth area of 0.32 cm^2 and a volume of 360 µL. For experiment 11 I used in-house developed microfluidic chips, consisting of 4 well compartments with a diameter of 4.5 mm, interconnected with micro-sized, axon-permissive tunnels with a length of 700 µm (de Wijdeven et al., 2018). The volume per cell compartment in these microfluidic chips is 130-200 µL. For experiment 13, 6-well Microelectrode arrays (MEAs) 60-6wellMEA200/30iR-Ti (Multichannel system) were used, containing a volume of approximately 500 µL per well, with 9 electrodes in each well.

2.2.1 MAIN CHANGES BETWEEN PROTOCOLS IN TERMS OF CHEMICALS AND COATING SUBSTRATE

In the first experiment, suspension media contained of 50:50 neuronal cell culture medium (NCCM) and dispase, supplemented with 1% Fetal Bovine Serum (FBS) and 1% penicillin-streptomycin (PS), where Corning matrigel was used as a coating substrate. Small tweaks of the protocol were done for the second experiment, increasing the concentration of FBS to 10% of total volume, and adding 1% L-glutamine to the cell suspension. In the third experiment, 1:1000 rock inhibitor (RI) was added to the cell suspension and using astrocytes as a feeder-layer was explored in addition to 3D-culture, i.e. EC layer II explant with 50:50 matrigel and seeding cell media. In the following experiments, protocols for cell media remained the same, but Poly-L-Ornithine (PLO) combined with laminin and Fibronectin were used as coating substrates. From experiment 6, neuronal cell culture media and L-glutamine supplement were exchanged with Neurobasal plus Medium supplemented with GlutaMAX™ and B-27 supplement. In addition, Poly-D-Lysine (PDL) was used as coating substrate, and penicillin-streptomycin (PS) was excluded from the protocol. In experiment 7, no further changes in the protocol were made, except adding DNase I to the cell media. The same protocol as in experiment 6 was used in experiment 8, with exception from 8 well Ibidi chip as coating vessel. In experiment 9, the only change was using geltrex with DMEM/F-12 as coating substrate. In experiment 10, 0.5% PS was added again to the protocol, but in further experiments 10-14 excluded again. In experiment 11, 13 and 14 same protocol regarding cell media as in experiment ten was used, though, PLO combined with laminin was used as coating substrate, where astrocytes were pre-plated to create a feeder layer. In experiment 12 the same protocol as in experiment 6 was used.

Table 3. Materials and chemicals used in protocols.

Product	Producer	Catalog/product number
Hibernate™ -A Medium	Gibco™	Cat# A1247501
Hanks' Balanced Salt Solution	Gibco™	Cat# 14170112
Neuronal Culture Medium	Thermo Scientific™	Cat# 88283
Neurobasal Plus Medium	Gibco™	Cat# A3582901
Dispase II	Gibco™	Cat# 17105041
Y-27632 (Dihydrochloride) RHO/ROCK pathway inhibitor	STEMCELL™ Technologies	Cat# 72302
Penicillin streptomycin	Gibco™	Cat# 15070063
L-glutamine	Gibco™	Cat# 25030081
Deoxyribonuclease I (DNase I)	Invitrogen™	Cat# 18047019
GlutaMAX™ Supplement	Gibco™	Cat# 35050061
B-27™ Plus Neuronal Culture System	Gibco™	Cat# A3653401
Poly-D-Lysine	Gibco™	Cat# A3890401
Poly-L-Ornithine	Sigma-Aldrich	Cat# P4957
Laminin	Gibco™	Cat# 23017015
Corning Matrigel Matrix	Life sciences	Cat# 354234
Geltrex™ LDEV-Free Reduced Growth Factor	Gibco™	Cat# A1413202
Rat Primary Cortical Astrocytes	Gibco™	Cat# N7745-100
Fibronectin	Sigma-Aldrich	Cat# F4759
Dulbecco's Phosphate-Buffered Saline	Gibco™	Cat# A1285801
Trypan Blue Stain	Invitrogen™	Cat# T10282
Corning® 96 well plates	Sigma-Aldrich	Cat# CLS9102
Corning® 12 well plates	Sigma-Aldrich	Cat# CLS3512-100EA
6-well Microelectrode array	Multichannel systems	60-6well MEA200/30iR-Ti
Microfluidic chips	In-house developed.	*
8 Well chamber	Ibidi® Cells in focus	Cat# 80841

*In-house developed by van de Wijdeven et al., 2018.

2.2.1 CELL IDENTIFICATION

To consistently identify LEC-layer II and CA3 pyramidal neurons in fresh unstained tissue I first underwent extensive training with my supervisor on how to recognize salient features similar to those established by cytoarchitectonic studies (see section 1.5 EC anatomy). Recognizing salient features was achievable on fresh tissue when viewed under a stereoscope using fiber optic lamps angled to give appropriate contrast. In order to ensure that I did not drift from the established

criteria, I regularly revisited in-house Nissl stained horizontal sections and viewed these under a microscope (Zeiss), comparing these to the established criteria for delineation of EC as discussed in section 1.5 EC anatomy. If I ever was in doubt about the borders of the layers during the microdissection, the Paxinos Mouse Brain Atlas in addition to in-house delineations made by my supervisor and me was used to double-check borders at the given anatomical location in the given brain section (Paxinos & Franklin, 2012).

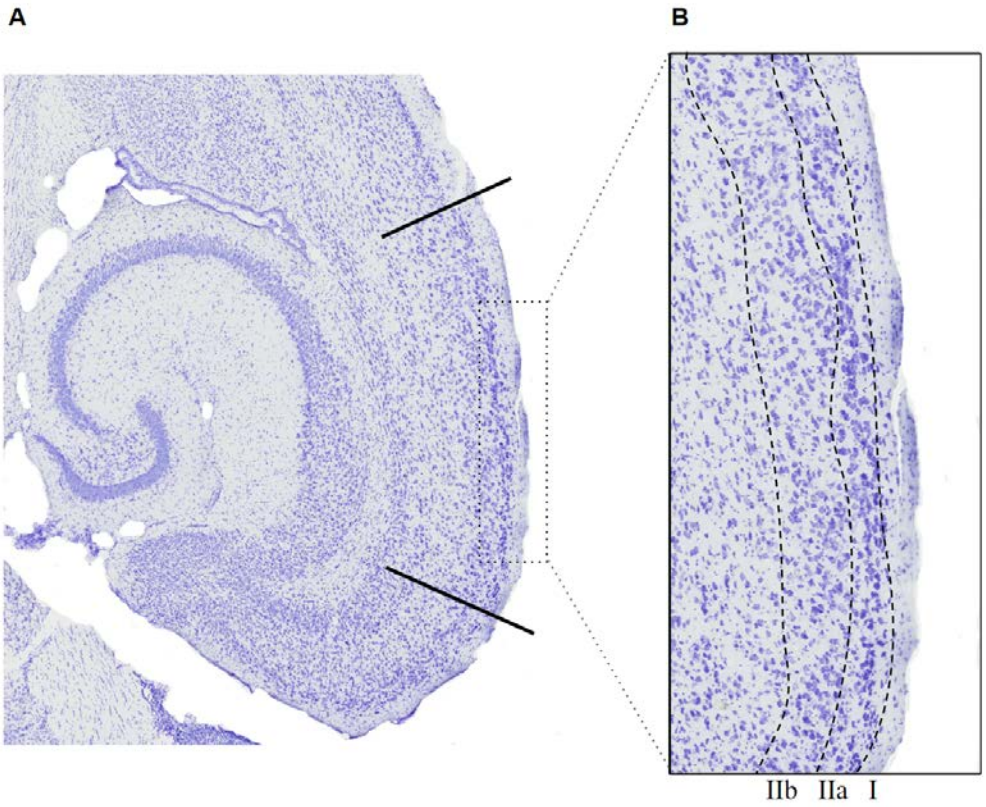

FIGURE 7. DELINEATION OF LATERAL ENTORHINAL CORTEX LAYER II.

(A) Photomicrography of a Nissl stained horizontal section from mouse brain. Area within the two borders indicate lateral entorhinal cortex (LEC). **(B)** Enlarged inset from (A) showing delineations of superficial layers IIa and IIb of LEC. *I would like to thank Arthur Laja for providing the photomicrograph.*

3.0 RESULTS

3.1 MAIN FINDINGS

My results show that culturing of LEC layer II-neurons harvested from young adult animals is possible. Across all fourteen protocols carried out in this thesis, a generally positive development was observed from experiment 6, judging by the increment in neuronal viability after plating of the neurons (Fig. 10). In terms of initial neuronal survival, a cell count was performed in ten out of fourteen experiments before plating of neurons (Fig. 8). Here, an increasing trend in results was observed from experiment 4-9, before a decreasing fashion was apparent in the remaining experiments 10-14.

In the five first experiments, good sections were obtained during dissection, and I was able to dissect out a substantial portion of LEC layer II. Based on visual inspections, dissociation of tissue and plating of neurons appeared successful. However, no attachment of neurons was observed one week after plating in either of the experiments. Further, in experiment 6 and 7, a drastic improvement was achieved where neuronal viability increased, and neurons survived up to two weeks after plating (Fig. 10-11). Regression arose in the following experiments 8 and 9, where no neuronal attachment was observed one week after plating of neurons. However, in experiments 10-14 neuronal viability increased, neuronal attachment and neurite outgrowth was achieved, and in experiment eleven and thirteen re-establishment of neuronal connections was present and growth cones could be observed (Fig. 12-14).

3.2 INITIAL NEURONAL SURVIVAL BEFORE PLATING

With exception from experiment 1, 2, 3 and 8, cell count was performed in all experiments, and initial neuronal survival (INS) before plating was observed (Fig. 8). Results from the cell counts show a tendency to increase until experiment 9, before a declining trend is expressed throughout the five restraining experiments. Results presented in Fig. 8 are calculated from two separate cell counts (A and B), where the average of these calculations are presented in Fig. 8 (see appendix 4 for results from cell count A and B).

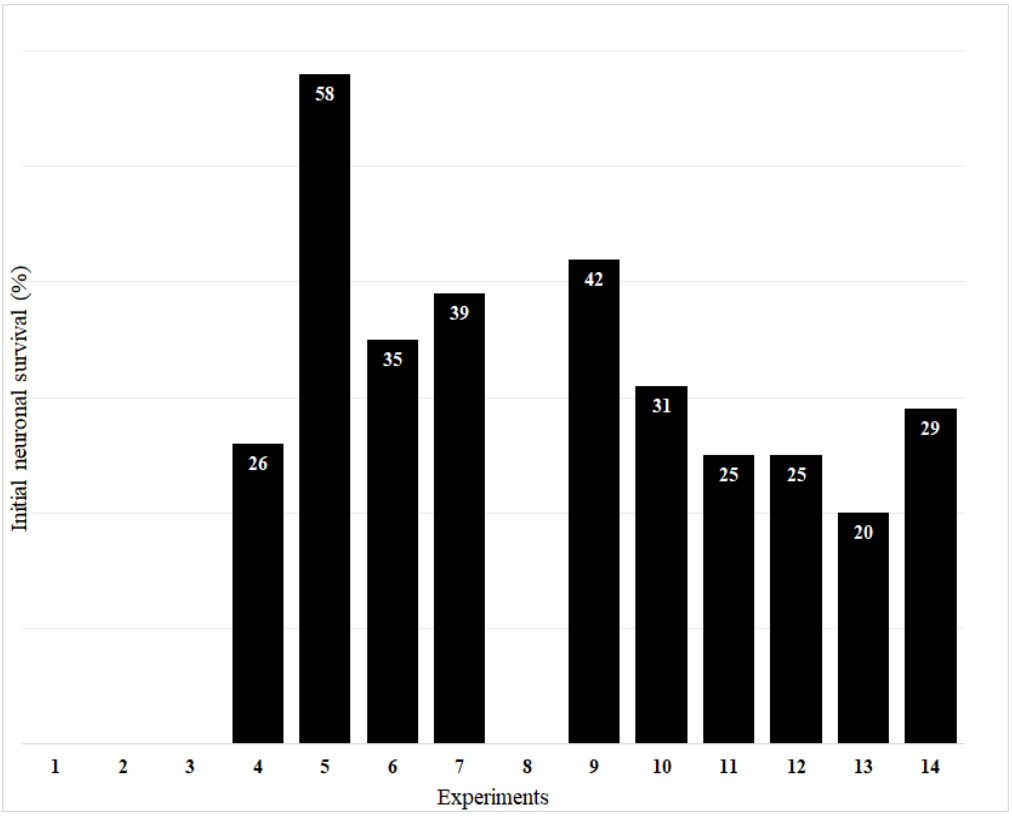

FIGURE 8. INITIAL NEURONAL SURVIVAL OF LEC LAYER II-NEURONS.

(X-axis) Results from all fourteen experiments can be observed on the x-axis (with exception from experiment one, two, three and eight where no cell count was performed). **(Y-axis)** Initial neuronal survival (%) of LEC layer II-neurons. Data points at each chart is showing the INS in the given experiment.

We estimated that the decreasing trend in INS from the cell counts could have a connection with the increase in age of animals used in the last five experiments. When comparing INS to the age of animals (AOA) a negative correlation (R=-.564) was observed, meaning that with increasing age of the animal, a decrease in initial neuronal survival is seen (Fig. 9). However, this negative correlation was not statistically significant (p=.089), and I interpret it as a trend indicating that substantially above the ages for animals used in this thesis may prove highly challenging.

If excluding experiments where INS was not calculated (experiment 1, 2, 3 and 8), four of the experiments contained tissue harvested from two animals (experiment 4, 5, 10 and 14), where the restraining experiments contained tissue from one animal. When comparing mean INS between these two groups, a mean INS of 36% is observed in the group containing tissue from two animals, whereas a mean INS of 32.2% is seen in the group containing tissue from one animal. Thus, a

small difference is displayed favoring the group containing tissue from two animals. Further, when comparing mean INS from experiments using animals grouped in AD (experiment 4, 6, 7, 10, 11 and 12) vs control (5, 9, 13 and 14), results show a mean INS of 31% and 39%, respectively. Interestingly, when comparing mean age and mean INS in the AD-group to the control group, we observed that in the AD-group both mean age (P57) and mean INS (31%) is lower compared to the mean age (P62) and mean INS (39%) in the control-group where both parameters are higher. This shows a conflicting result to Fig. 8, where increased age was correlated with a decreased INS.

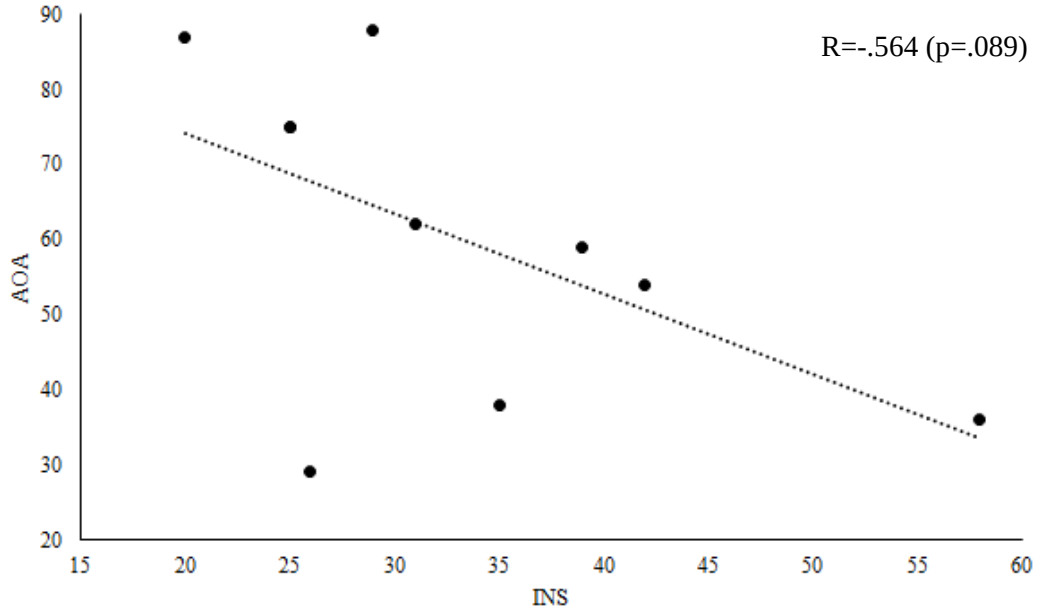

FIGURE 9. CORRELATION BETWEEN INITIAL NEURONAL SURVIVALS (INS) COMPARED TO AGE OF ANIMALS (AOA).

Initial neuronal survival (INS) in % on the x-axis is plotted against the age of animals (AOA) in weeks on the y-axis. Results show a non-significant (p=.089) negative correlation (R=-.564) between the two variables.

CA3 pyramidal neurons were plated along with LEC layer II-neurons in experiment 5, 11 and 13, where results from cell count were 36%, 10% and 13% viability, respectively. This result in an overall mean INS of 19.6% from cell counts performed on CA3 pyramidal neurons. This overall mean INS of 19.6% is radically lower relative to overall mean INS of 33% in LEC layer II-neurons.

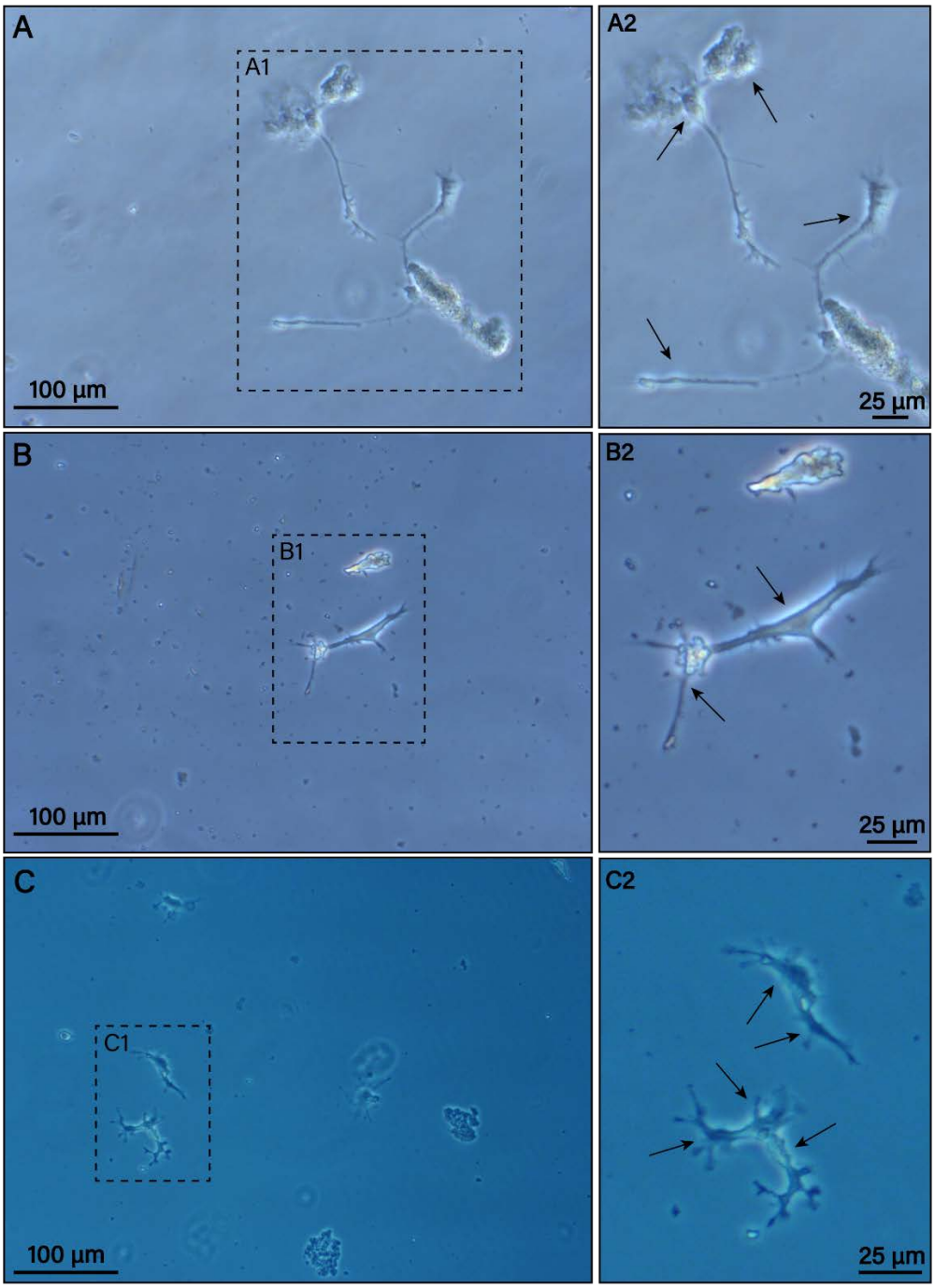

FIGURE 10. SMALL NETWORK FORMATIONS IN LEC LAYER II-NEURONS.

All images are taken from experiment six with a 10x objective on a microscope. Scale bar as indicated in all images and insets. **(A)** Image is taken eight days after plating of neurons. A2 is an enlarged inset from A1 where soma and neurites can be observed indicated by the arrows. **(B)** Image is taken eight days after plating of neurons. B2 is an enlarged inset of B1 where the soma of a neuron is indicated by the top arrow, while the bottom arrow indicates a neurite. **(C)** Image is taken ten days after plating of neurons. C2 is an enlarged inset of C1 showing neuronal attachment of five neurons indicated by the arrows. Connections between neurons can be observed as two small networks of two and three neurons.

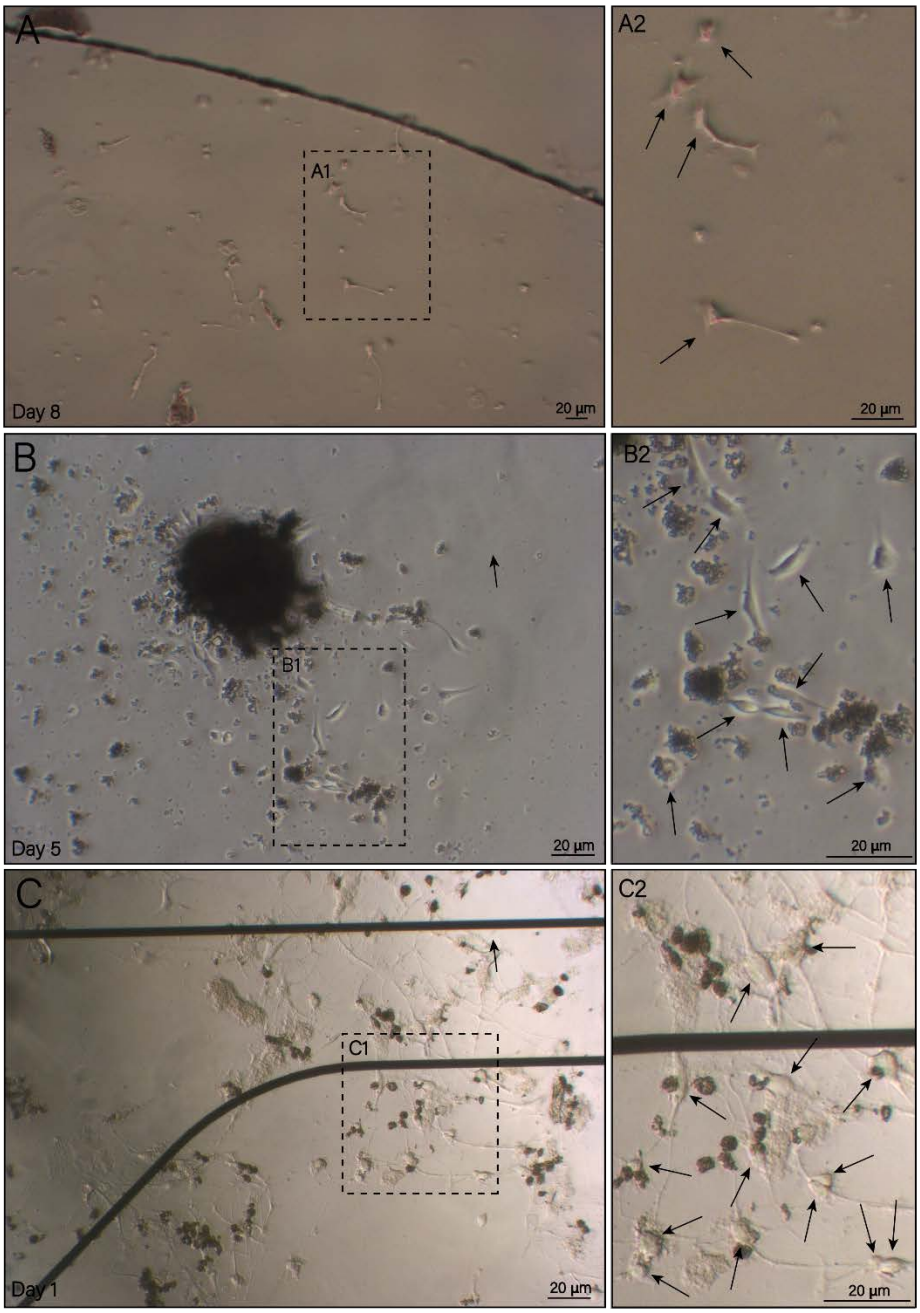

FIGURE 11. PROGRESSION IN VIABILITY OF LEC LAYER II-NEURONS.

Scale bar as indicated in all images and insets. **(A)** Image is taken from experiment six with a 10x objective on microscope eight days (Day 8) after plating of neurons. A2 is an enlarged inset of A1 showing neuronal attachment of four neurons indicated by the arrows. **(B)** Image is taken from experiment twelve with a 20x objective on microscope five days (Day 5) after plating of neurons. B2 is an enlarged inset of B1 showing neuronal attachment of nine neurons indicated by the arrows. **(C)** Image is taken from experiment eleven with a 20x objective on microscope one day (Day 1) after plating of neurons. C2 is an enlarged inset of B1 showing neuronal attachment of fourteen neurons indicated by arrows.

3.3 NEURONAL VIABILITY AFTER PLATING

3.3.1 MAIN IMPROVEMENT IN NEURONAL VIABILITY WAS DUE TO CHANGES IN MEDIA COMPOSITION

After adjustments of the protocol in terms of media composition in experiment six, drastic improvement of results was observed. From this time point, Neurobasal plus medium (Gibco, Cat# A3582901) supplemented with B-27 plus supplement (Gibco, Cat# A3653401) and GlutMAX supplement (Gibco, Cat# 35050060) was included in the protocol, replacing Neuronal culture medium (Thermo Scientific, Cat# 88283) and L-glutamine supplement (Gibco, Cat# 25030081). In addition to this media change, PS was excluded from the protocol.

When comparing INS in experiments 1-5 before-, and to experiments 6-14 after this media change, results from experiments before change of media composition show a mean INS of 42%, whereas mean INS in experiments after this change was 27%. In spite of this decline in INS, results that were achieved after plating of neurons were undoubtedly improved in experiments after the inclusion of new media compared to those before. Results after plating of neurons showed that neuronal viability was clearly increased, as neurons survived up to two weeks after plating, and neuronal attachment was obtained where the formation of small anatomical networks was observed (Fig. 10-14).

3.3.2 IMPROVEMENT IN NEURONAL ATTACHMENT AND NEURITE OUTGROWTH WITH ASTROCYTES AS A FEEDER LAYER

In experiment 11 and 13, coating plates was pre-plated with astrocytes (Gibco, Cat# N7745-100) as a feeder layer on top of the coating substrate Poly-L-Ornithine (Sigma-Aldrich, Cat# P4957) and Laminin (Thermo Scientific, Cat# 23017015). Results from these two experiments stand out from other experiments as neurons attached to the growth substrate and neurite outgrowth were observed already one day after plating of LEC layer II-neurons (Fig. 13). Same results were achieved with CA3 pyramidal neurons in experiment eleven, where neuronal attachment and neurite outgrowth was observed (Fig. 12A and 12D). In addition, growth cones extending from LEC layer II- neurons were observed five days after plating in experiment 11 (Fig. 13), which previously had not been observed in experiments where astrocytes not were included as a feeder

layer. Astrocytes were also plated as a feeder layer without any coating substrate in experiment 3, however, no neuronal attachment was obtained, and neurons aggregated and died within one week after plating.

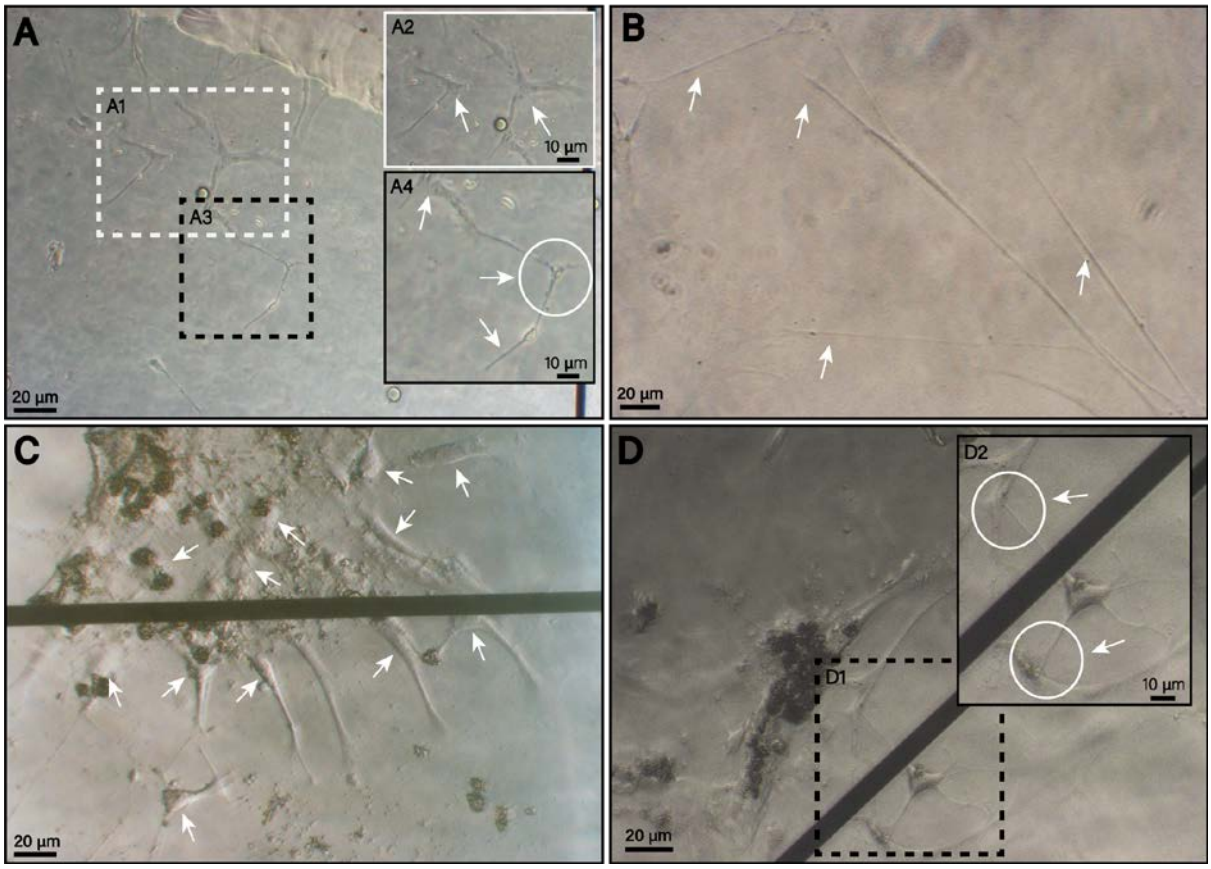

FIGURE 12. NEURONAL ATTACHMENT OF LEC LAYER II-NEURONS AND APPARENT STRUCTURAL CONNECTIONS BETWEEN CA3 PYRAMIDAL NEURONS.

Images from A and B is taken from experiment eleven, while imaged from C and D is taken from experiment thirteen. All images are taken with a 20x objective on a microscope. Scale bar as indicated in all images and insets. **(A)** Image is taken four days after plating of neurons. A2 is an enlarged inset of A1 showing neuronal attachment of two neurons indicated by the arrows. A4 is an enlarged inset of A4 where the middle arrow pointing towards the circle show connection between two neurons which are indicated by the other two arrows. **(B)** Image is taken five days after plating of neurons, and show neurites extruding from LEC layer II-neurons indicated by arrows. **(C)** Image is taken four days after plating and show twelve LEC layer II-neurons indicated by arrows. **(D)** Image is taken four days after plating and show network connection between LEC layer II-neurons indicated by the two arrows pointing at two circles.

3.3.3 MINOR ADJUSTMENTS IN PROTOCOL POTENTIALLY INCREASING NEURONAL VIABILITY

As a considerable number of neurons tended to aggregate and die in nearly all experiments, it was aimed from experiment 11 onwards to plate only isolated neurons, and not include all chunks of tissue which had been conducted in previous experiments. This was done to lower the amount of dead tissue and neurons at plating that possibly could contribute to induce apoptotic factors in the culture. Debris was still present in experiments 10-14; however, neuronal viability was highly improved in experiment eleven and thirteen compared to previous experiments.

Poly-D-Lysine (PDL; Gibco, Cat# A3890401) was explored as a coating substrate in experiment 6, 7, 8, 12 and 14. Neuronal attachment was observed in all experiments where PDL was included as a coating substrate and could, therefore, enhance neuronal viability in LEC layer II-neurons as neuronal attachment not was observed when using either matrigel (Life sciences, Cat# 354234), fibronectin (Sigma-Aldrich, Cat# F4759) or geltrex (Gibco, Cat# A1413202) as coating substrates. Survival of neurons was also improved when using Poly-L-Ornithine (PLO; Sigma-Aldrich, Cat# P4957) and Laminin as the main coating substrate in experiment 11 and 13, where neuronal attachment and extruding neurites were observed.

Following an issue in experiment 8, where the tissue was sticking to the pipette tip, all pipette tips and containers were pre-rinsed with media before handling of tissue. This adjustment led to no further problems regarding tissue loss due to sticking to equipment material and could, therefore, be a contributing factor to the observed increase in neuronal viability throughout experiment 10-14.

Improved aseptic techniques were implemented from experiment 6, where all surgical equipment was autoclaved or washed with 70% ethanol. In addition, time spent during the process of dissection, disaggregation-, and plating of neurons was reduced by up to 1.5 hour from the first experiment to experiment 6.

To lower the grade of media evaporation all culture dishes were sealed with Parafilm from experiment 6. In addition, neurons were plated in the middle wells of multi well-plates surrounded by sterile water, and an extra petri-dish with sterile water was placed along with plated microfluidic chips and MEAs in experiment 11 and 13.

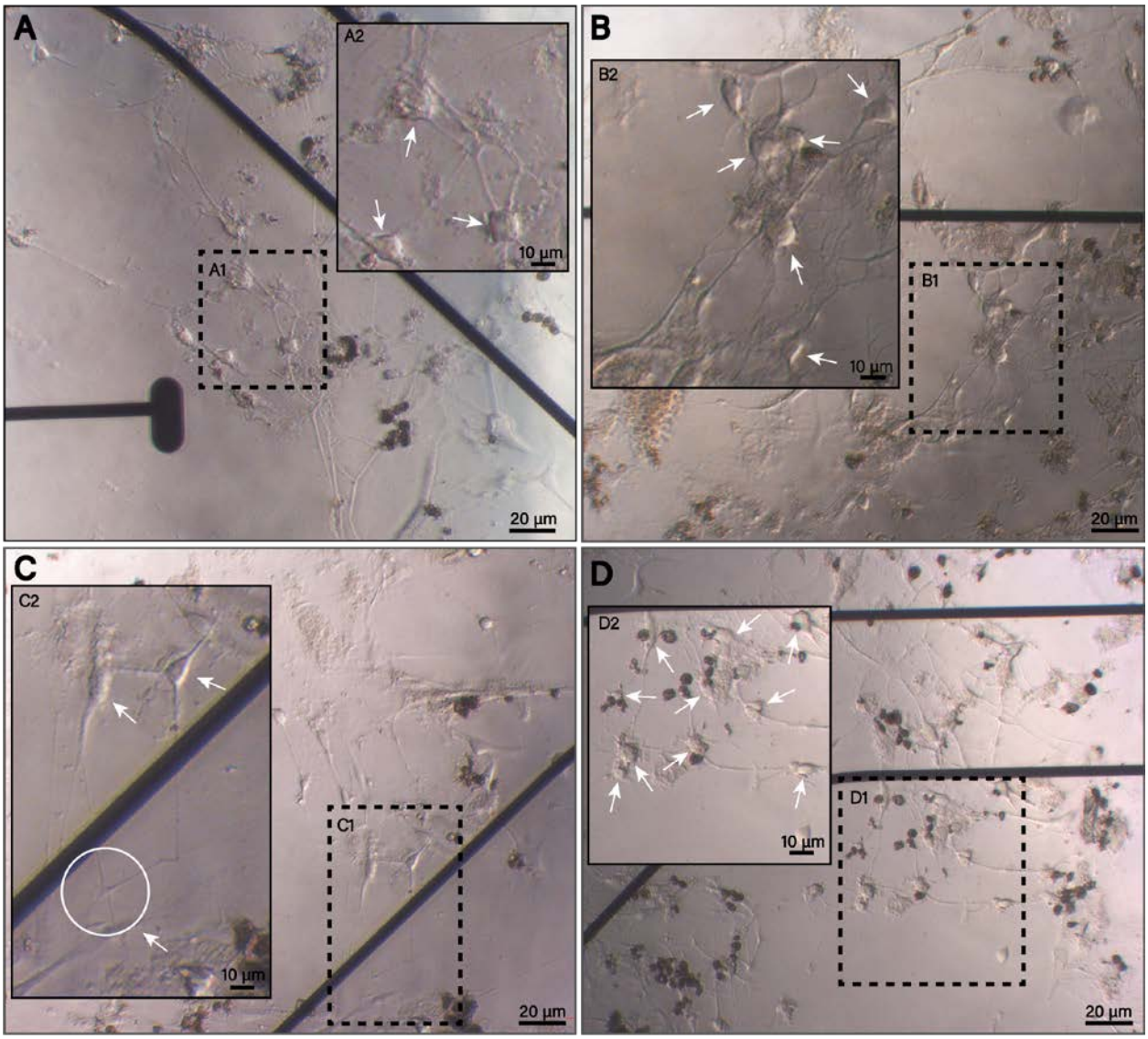

FIGURE 13. NEURONAL ATTACHMENT AND NETWORK CONNECTIONS BETWEEN LEC LAYER II-NEURONS.

All images are taken from experiment eleven with a 20x objective on a microscope one day after plating of neurons. Scale bar as indicated in all images and insets. **(A)** A2 is an enlarged inset from A1 showing neuronal attachment of three neurons indicated by the arrows. **(B)** B2 is an enlarged inset of B1 showing neuronal attachment of six neurons indicated by arrows. **(C)** C2 is an enlarged inset of C1 showing neuronal attachment of two neurons indicated by the two top arrows, and neurite connections indicated by the bottom arrow pointing at the circle. **(D)** D2 is an enlarged inset of D1 showing neuronal attachment of two ten neurons indicated by the arrows.

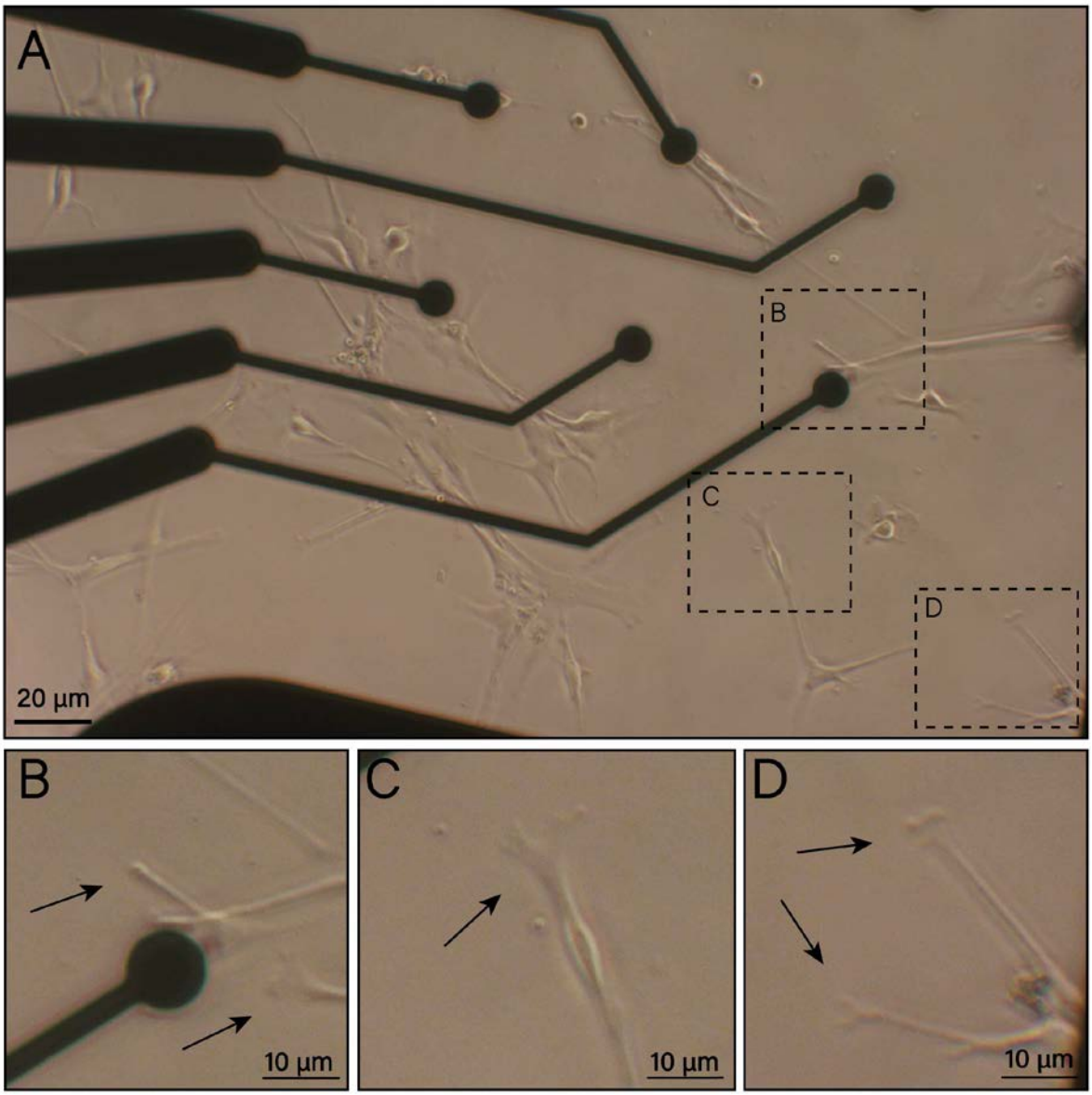

FIGURE 14. GROWTH CONES EXTRUDING FROM LEC LAYER II-NEURONS.

(A) Image is taken from experiment thirteen with a 20x objective on a microscope five days after plating of neurons. **(B-D)** Enlarged insets from A where arrows indicate growth cones on neurites extruding from LEC layer II-neurons. Scale bar as indicated in all images and insets.

4.0 DISCUSSION

4.1 SUMMARY OF MAIN FINDINGS

The work presented in this thesis aimed to establish an LEC layer II-neuron culture using freshly dissected neurons from young adult APP/PS1 mice and controls. Here, fourteen different experiments were performed in order to develop an optimal protocol being able to achieve high neuronal viability. My results demonstrate that it is possible to 1) identify LEC layer II-neurons on fresh vibratome sections, 2) selectively dissect these out while maintaining tissue integrity, and 3) dissociate and culture such neurons to the point that they start self-organizing into new networks i.e. forming structural connections (Fig. 13-14). In the five first experiments, neurons tended to aggregate and die within a week after plating. As the protocol was increasingly optimized increased neuronal viability was achieved, beginning from experiment 6. The best results were achieved in the last four experiments with a substantial number of neurons attached to the coating substrate and re-formed structural connections were observed (Fig. 12-13). In the last four experiments, we also observed growth cones extruding from neurites (Fig. 14). These improvements of neuronal viability and display of dynamic behavior associated with connectivity are highly important, and, not least, necessary for future study of such neuron-preparations at a structural and functional level *in vitro*. Here, we observed increasing complexity of the LEC layer II-neurons *in vitro*, where the development of structural connections was achieved. A secondary aim was to also investigate functional neuronal connections of the LEC layer II neurons using MEAs. However, by the time I had brought the neuronal viability to a level allowing this an incident of contamination occurred, preventing these experiments within the given timeframe.

4.2 DISCUSSION OF RESULTS AND CULTURING STRATEGIES

My results from this thesis demonstrate that it is possible to obtain viable LEC layer II-neural neurons *in vitro* through identification, dissection and culturing of these specific neurons using optimized protocols. As neurons in LEC have been demonstrated to be particularly vulnerable in the initial stages of AD in terms of neuronal loss (Gomez-Isla et al., 1996; Kordower et al., 2001), electrophysiological firing properties (Marcantoni et al., 2014), and impaired metabolism (Khan et al., 2014), being able to identify, selectively collect and culture neurons from LEC layer II is

important as it may enable a new level of investigation related to dysfunctions in these highly AD-relevant neurons.

Maintenance of tissue integrity while dissecting out neurons is crucial to preserve high neuronal survival. Here, we observed this neuronal survival by performing cell counts and thereby assessing the initial neuronal survival (INS), as an indicator of the integrity of the dissected neuronal population. An increasing trend in INS was observed until experiment 9 before a decline in INS was observed from experiment 10-13, subsequently increasing again in experiment 14. We assumed that this trend could be due to the increased age of animals, which was used in experiment ten to fourteen. As seen in Fig. 9, there was no statistically significant correlation ($p=.089$) between the age of the animal and the initial INS. The possibility of differential vulnerability between the animals used here as a function of age should thus be interpreted with caution. For example, in the AD-mice group the main age of the animal (P57) and the main initial survival (INS; 31%) was lower compared to controls (mean age P62, mean INS 39%), which conflict with the observed trend in Fig. 8 where increased age is correlated with decreased initial neuronal survival (Fig. 9). Note that also, in the AD group tissue was taken from one animal was used in five out of six experiments, whereas in the control group, all experiments included tissue from two animals. As mean INS was higher in experiments with tissue from two animals (36%) compared to those with tissue from one animal (32.2%), this might be related to the observed difference in AD-mice vs. controls. Irrespectively of this, the observed trend with increased INS until experiment nine, before a decreasing trend was apparent did not affect the neuronal viability after plating of neurons.

To provide a favorable microenvironment to the neurons, the media composition is a way of providing nutrients that will optimize the energy supply required by the neurons. *In vivo*, the microenvironment is controlled by essential- and non-essential amino acids provided to the neurons by synthetization of proteins and through diet, in addition to various salts, glucose and blood serum (as reviewed by Lodish et al., 2000). *In vitro*, all these components need to be provided by a cell media composition for optimal culture conditions, and the importance of this was demonstrated here.

When comparing INS in experiments before and after including Neurobasal plus medium (NPM) supplemented with B27 plus supplement and GlutaMAX supplement in the protocol, INS before inclusion has a mean INS of 42%, while mean INS after inclusion was 27%. Even though mean

INS was higher before inclusion of NPM, neuronal viability in experiments with NPM supplemented with B-27 and GlutaMAX was undoubtedly improved as neurons attached to the coating substrate and survived up to two weeks after plating. This indicates that the composition of cell media has a decisive effect on neuronal viability of LEC layer II-neurons, given that before inclusion of NPM, no neuronal attachment was achieved, while after inclusion, a high increment of neuronal viability and neuronal attachment was achieved. In previous results from Brewer (1997), they observed improved neuronal viability with 30% increment four days after plating primary cortical neurons from adult mice when including B-27 supplement in dissociation and plating of neurons, compared to using only NPM without B-27 supplement. This indicates that not NPM alone, but NPM supplemented with B27 increases neuronal viability after plating of neurons. In this thesis, B-27 was included in all protocols from experiment 6, and could, therefore, be a decisive factor leading to the increased neuronal viability that was observed from experiment 6. These improvements in the results after the inclusion of NPM with B-27 supplement can also be seen in neurons co-cultured with astrocytes, where in experiment 3 before NPM was included, no neuronal attachment was achieved, whereas in experiment 11-13 when NPM was included, neuronal attachment was achieved already one day after plating of neurons.

Another parameter potentially affecting the neuronal viability of LEC layer II-neurons is the presence of antibiotics in the cell culture. In all experiments where the antibiotic penicillin-streptomycin (PS) was included in the protocol (experiments 1-5 and 10), no neuronal attachment was achieved one week after plating of neurons. Antibiotics have previously been demonstrated to impair neuronal growth rate in embryonic stem cells (Cohen et al., 2006), affect interference of excitability and electrophysiological properties in primary hippocampal neurons (Bahrami & Janahmadi, 2013), enhancement of cell proliferation of eukaryotic cells (Cooper, Laxer & Hansbrough, 1991), and altering gene expression in human liver cell lines (Ruy et al., 2017). As antibiotics have been demonstrated to impair different parameters in other cell cultures and cell lines, antibiotics cannot be excluded as a contributing negative decisive factor for neuronal viability of LEC layer II-neurons.

Further, astrocytes used as a feeder-layer seemed to play a decisive role for neuronal viability of LEC layer II-neurons. Astrocytes are important to neurons *in vivo* as they support neuronal migration, enhance growth and arborization of dendrites, and regulate synaptic development and

pruning (Farhy-Tselnicker & Allen, 2018). Furthermore, astrocytes contribute to the stabilization of the neuronal network. *In vitro*, the astrocytes can increase neuronal viability as they facilitate attachment of neurons by working as a feeder layer where they can supplement the biological environment with growth factors and metabolites (Freshney, 2016, p. 114). Astrocytes were plated as a feeder layer in experiment 3, but one week after plating of LEC layer II-neurons, no neuronal attachment was observed. However, when astrocytes were plated as a feeder layer in experiment 11-13, high neuronal viability were achieved where neuronal attachments was achieved one day after plating of neurons, and after three days outgrowth of neurites and growth cones was observed (Fig. 12-14). This increasing effect of neuronal viability in experiment 11 and 13, where astrocytes were included as a feeder layer, could be a result of the change of the media composition that was conducted from experiment 6. Another contributing factor to why neuronal viability was improved in experiment 11 and 13 could also be due to the coating substrate PLO combined with Laminin, which was used in experiment 11 and 13. In accordance with results from Noble and colleagues (1984), neurons plated on astrocytes as a feeder layer generally grew as single neurons and did not tend to aggregate to the same extent as neurons in cultures where astrocytes not was included as a feeder layer.

Most neurons require attachment for growth, and thus, an adherent cell culture is required (Lodish et al., 2000). In the neuronal network *in vivo*, the extracellular matrix (ECM), which is composed of substances secreted from the cells itself, ensures physical scaffolding for the neurons as it helps binding the tissue together (Frantz, Stewart & Weaver, 2010). Similar cell adhesion is crucial to obtain *in vitro* by using a coating substrate that facilitates neuronal attachment to the culture vessel and promotes neurite extension, so that neuronal connections can be established (Lodish, et al., 2000). Here, neuronal attachment was only achieved in experiments where Poly-D-Lysine (PDL) and Poly-L-Ornithine (PLO) combined with Laminin was used as coating substrate; thus, the coating substrate seems to be a critical factor for viability of LEC layer II-neurons *in vitro.* This can be seen as the exact same protocol was used in experiment 6 and 9, except from the fact that PDL was used as coating substrate in experiment 6, while Geltrex was used as coating substrate in experiment 9. Results from experiment 6 showed neuronal attachment and neuronal survival up to fourteen days after plating, whereas no neuronal attachment was observed in experiment 9 up to one week after plating. Further, when using PLO + Laminin as the coating substrate in experiment 4 and 5, no neuronal attachment was obtained. This underscored that the coating substrate can be

decisive for neuronal attachment of LEC layer II neurons. Also, note that since both PDL and PLO contain polymers, the increased neuronal attachment and neurite outgrowth could be related to the surface charge (Freshney, 2016, p. 113).

Plating density in cultures can be crucial to neuronal viability, and the ability for neurons to form structural connections is highly determined by the density (Biffi et al., 2013). No neuronal attachment was observed in experiment 1-5, or 8-9, and a common denominator for all these experiments is that an 8-well Ibidi chip with a vessel volume of 400 µL per well was used as coating vessel. In addition, chunks of tissue was plated along with the dissociated neurons in experiment 1-5 and 9. If the culture vessel is too small compared to the amount of plated neurons, overcrowding of neurons can induce apoptotic- and in worst case, necrotic factors (Qiao & Farrell, 1999). In the contrary, only one chunk of tissue along with dissociated neurons was plated in experiment 8, though, the natural explanation for to why the viability was so low could be due to the fact that the tissue being extremely sticky. This latter feature is commonly experienced in tissue that has entered the first step of decomposition. In both experiment 10 and 12, 96-well culture vessel with a volume of 360 µL per well was used. In experiment 10, chunks of tissue was plated along with the dissociated neurons, and no neuronal attachment was observed. However, the antibiotic PS was also included in the protocol, which previously discussed could be a decisive factor. Whereas in experiment 12, only one chunk of tissue was plated along with dissociated neurons, in addition astrocytes was plated as a feeder layer, and here, neuronal attachment was observed. In experiments 11 and 13, culture vessel volume was 130-200 µL and 500 µL was used, respectively. Here, in both experiments only dissociated neurons were plated and astrocytes was plated as a feeder layer, where results showed neuronal attachment already one day after plating of neurons (Fig. 13). When looking at experiments 6, 7 and 14, 12-well plates with a total volume of 6.9 mL per well was used in all experiments and neuronal attachment was observed from all three experiments. Based on these findings from all experiments, the coating vessel itself do not seem to be a decisive factor for neuronal attachment and viability, however, neuronal density at plating along with other parameters like astrocytes as a feeder layer, media composition and coating substrate do seem to be highly important being able to reach high viability of neurons.

Two different neuronal types were explored in this thesis; LEC layer II-neurons and CA3 pyramidal neurons. My results show that it is possible to achieve neuronal attachment in both types

from young adult mice *in vitro.* Furthermore, results show that CA3 pyramidal neurons had lower initial neuronal survival (INS) pre-plating (main INS 19.6%) compared to LEC layer II-neurons (mean INS 33%), and viability after plating was lower in CA3 pyramidal neurons based on own observations after visual inspection under the microscope. Therefore, CA3 pyramidal neurons could with advantage have been cultured using a separate protocol to adapt the conditions optimally. However, as the main aim of this thesis was to establish a culture on LEC layer II-neurons, focus was placed into adjusting the protocol favoring these neurons.

4.3 METHODOLOGICAL CONSIDERATIONS

Another trivial factor possibly affecting my results is that by completing several protocols, and thus gaining experience, I became increasingly efficient in carrying out each individual experiment. This may have led to less exposure of the tissue to open air, decreased risk for contamination and the ability to hold the tissue more consistently at a low temperature.

Following vibratome of tissue, an issue regarding incomplete brain sections was apparent, where the brain tissue tended to crumble, with the most dorsal part of the brain being particularly affected. In addition, brain sections tended to break (regardless of the anatomical plane) at superficial layers, which is unfortunate as parts of the LEC got destroyed. This leads to less tissue available for culturing, effectively reducing the number of dissociated neurons pre-plating. These issues could be due to the quality of the given animal's brain tissue, but, more likely they could be due to the time spent during brain extraction and dissection, as brain tissue will inevitably begin to disintegrate following from removal of its natural environment. Other factors contributing to breakage at superficial layers may be because too much glue was used to attach the brain to the vibratome stage, or that the brain was attached suboptimally, such that sections were cut unevenly leading to partial loss of superficial layers. Another factor contributing to incomplete brain sections is the quality and accuracy performed during fresh dissection of the brain. Abrupt removal of the scull can make the temporal bone penetrate the cortex, and great care must be taken when extracting the brain. Also, scissors and forceps used during dissection can easily cut into the brain tissue without leaving obvious marks, subsequently showing up upon having the vibratomed sections ready for microdissection. These issues can all be safe guarded against by focusing on quality and accuracy when dissecting brain tissue, although, extensive training will always be required to achieve the necessary skill level.

Neurons aggregating, and dying was a common problem across all experiments. In protocol 1-9, fully dissociated neurons were seeded along with chunks of tissue that were incompletely disaggregated. This was done to culture as many potentially viable neurons as possible. However, from experiment 11-14, with the exception of experiment 12, only fully dissociated neurons were plated and chunks of tissue were excluded in the highest degree possible. This was done since a high level of aggregating and dead tissue can result in high levels of apoptotic, or even necrotic factors spreading to other healthy living neurons resulting in accelerating overall neuronal death. In agreement with present findings, previous studies have reported that aggregation and lower viability of neurons occur if debris is apparent in the culture (Brewer, 1997; Eide & McMurray, 2005). The results from my thesis shows that when plating neurons onto small coating surfaces, it is best to exclude large chunks of tissue to lower the amount of debris affecting neuronal viability. However, for larger coating areas like the 12 well-plate, small pieces of non-dissociated tissue can be included as the amount of total media is much higher. Specifically, as the results from experiment 12 showed, neurons can expand from the incomplete disaggregated tissue explants (Fig. 10B).

Neuronal death and aggregation could also be due to the growth area (cm^2) of the culture dish as too large an area will prevent the neurons from forming connections with each other as they are too distant. On the other hand, if there is a too high concentration of neurons it can result in high levels of apoptotic, and in the worst case, necrotic factors spreading to other healthy living cells resulting in low viability and rapid cell death. The concentration of cells in the primary culture should be much higher than that normally used for subculture, because the proportion of cells from the tissue that survives in primary culture may be quite low. Time spent in both the anatomy lab and the cell culture lab for each experiment was reduced during the project period as my competency increased.

An incident of contamination occurred in the neuronal cultures in experiment 11-14 as they were performed in the same time period. There could be several reasons as to why this occurred. Contamination can occur from contaminated equipment like pipette tips and surgery equipment, contaminated media solution, dust particles etc. (Stacey, 2011). A specific factor that could be illuminated is the fact that PS was not included in any of the experiments that was affected (experiment 10-14), and contamination could therefore been to the lack of antibiotics. However,

contamination did not occur in experiments 6-10 where antibiotics not were included, and as the PS seemed to be affecting neuronal viability it was excluded from the protocol in experiment 11-14. Nevertheless, the lack of antibiotics can't be eliminated as a decisive cause of contamination in experiments 11-14. It is hard to pinpoint the exact reason to what caused the contamination, however, to prevent this in future experiments improved aseptic techniques and accuracy should be conducted.

In terms of animals included in this project, different age groups were used. This is a factor making it harder to compare differences in INS across experiments as there was a range of 60 days (P28-P88) between the youngest and the oldest animal included. Even though a non-significant correlation between age of the animal and INS were observed, some of the highest cell survivals were observed when younger animals were used in experiments. To the contrary, in the four last experiments, where the best results were obtained, animals with the highest age were used. In order to better compare results with the age of the animals, specific age groups could have been structured to get a better overview and thus see if there was a connection. Though, with regard to ethical considerations dealing with minimizing the number of animals used, we used animals in-house at the given time point each experiment was performed. This accounts for the different ages of the animals used.

Cell count was not performed in all experiments. This is unfortunate, as INS was not measured in four of the protocols, lowering the ability to compare factors like age of animals (AOA), final outcome and eventual differences in results between AD animals vs. control. Cell count was not performed due to focus on other techniques performed while culturing neurons in the first three trials, and as sticky tissue did not allow for it in experiment 8. In the same way, to better monitor results and compare eventual differences across experiments, images taken on the microscope should have been provided from each experiment.

4.5 FUTURE PERSPECTIVES

As high viability of LEC layer II-neurons was achieved and neurons formed new structural connections, the specific neurons' ability to re-connect and form *in vitro* functional networks should be further investigated by using microelectrode arrays (MEAs), with the aim of detecting potential differences in network excitability and synchrony between AD-neurons vs. normal controls. Further, to investigate the causative neuronal vulnerability in LEC layer II neurons,

physical manipulations regarding pharmacological and genetic manipulations can be conducted in *in vivo* cultures as different components easily can be added to the culture media to affect various cellular compartments like proteins, ion channels and receptors of interest (Carter & Shieh, 2015, p. 308). As results from this thesis evidence neuronal attachment of both LEC layer II-neurons and CA3 pyramidal neurons, a logical next step would be to make these different populations of neurons re-connect, which could be achieved using multi-well chambered microfluidic chips containing directional axon tunnels. A separate protocol for culturing CA3 pyramidal neurons should be implemented judging by results from initial neuronal survival, which was generally lower, compared to LEC layer II-neurons in this thesis. Based on visual inspections on the microscope, not as many CA3 pyramidal neurons attached to the growth substrate compared to LEC layer II-neurons.

5.0 CONCLUSIONS

The results from this thesis show that it is possible to establish an *in vitro* cell culture with freshly dissected LEC layer II-neurons from young adult animals. Taken together, the results show that it is possible to 1) identify LEC layer II-neurons on fresh vibratome sections, 2) selectively dissect these out while maintaining tissue integrity, and 3) dissociate and culture such neurons to the point that they re-form structural connections. Improved neuronal viability, increased neuronal attachment and re-formation of anatomical networks were achieved through systematically testing of fourteen different protocols. Four main factors seem to be of high importance improving neuronal viability of LEC layer II-neuronal cultures; 1) optimal culture conditions by media composition, 2) coating substrate, 3) astrocytes as a feeder layer, and 4) aiming to plate only fully dissociated neurons. By showing that it is possible to selectively identify, dissect out and culture neurons from this anatomical region, this thesis introduce the potential for future studies to further investigate the initial stages related to AD in these specific neurons.

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APPENDIX 1 – INDIVIDUAL PROTOCOLS FOR EACH EXPERIMENT

<table>
<tr><th colspan="2">PROTOCOL 1</th></tr>
<tr><td>Materials and reagents</td><td rowspan="6">Procedure
1. Transfer all tissue into a sterile 1.5 mL eppendorf tube containing the suspension media.
2. Incubate at 37°C with 5% CO2 for 20 minutes.
3. Aspirate excess media leaving the tissue in enough media so as not dry out, add 50 µL of dissociation media and triturate tissue 10 times with a 1000 µL pipette, avoid blowing air bubbles in the suspension.
4. Transfer cell suspension to a 15 mL Eppendorf tube and add suspension media up to 3 mL.
5. Centrifuge tissue at 200x g for 2 minutes.
6. Aspirate excess media and re-suspend the cell pellet in 100 mL fresh seeding media. Complete dissociation by triturating tissue with a 1000 mL pipet 10 times.
7. Divide the dissociated cells in four coated wells of an 8-well Ibidi chip.
1 well: incubated 15 min before seeding
1 well: explant culture not dissociated
2 wells: immediately seeding in wells
8. After 24 hours, aspirate half of the medium from each well and replace with fresh seeding media.</td></tr>
<tr><td>Dispase II (Gibco, Cat# 17105041)
NCM (Thermo Scientific, Cat# 88283)
FBS (Gibco, Cat# 16000044)
PS (Gibco, Cat# 15070063)
Matrigel (Life sciences, Cat# A1413202)</td></tr>
<tr><td>Equipment</td></tr>
<tr><td>1.5 mL Eppendorf tube
15 mL Eppendorf tube
Cell culture incubator
Centrifuge
8 well Ibidi chip
10-, 100- and 1000 mL pipet + pipette tips</td></tr>
<tr><td>Recipes</td></tr>
<tr><td>Dissociation media
50:50 Dispase and NCM, supplemented with 1% FBS and 1% PS.
Fresh cell media
NCM supplemented with 1% FBS and 1% PS.</td></tr>
<tr><td>Coating</td></tr>
<tr><td>1. Thaw pre-aliquoted corning matrigel on ice until one ice crystal is left. Spray with 70% ethanol and transfer to laminar hood.
2. Immediately use the diluted matrigel solution to coat plates.
3. Incubate at 37°C with 5% CO2 for 1 hour, or overnight at 4°C.
4. If culture plate has been stored at 4°C, allow the plate to incubate at 37°C for at least 30 min before removing the matrigel solution.</td></tr>
</table>

PROTOCOL 2

Materials and reagents

Dispase II (Gibco, Cat# 17105041)
NCM (Thermo Scientific, Cat# 88283)
FBS (Gibco, Cat# 16000044)
L-glutamine (Gibco, Cat# 25030081)
PS (Gibco, Cat# 15070063)
Matrigel (Life sciences, Cat# A1413202)

Equipment

1.5 mL Eppendorf tube
15 mL Eppendorf tube
Cell culture incubator
Centrifuge
8 well Ibidi chip
10-, 100- and 1000 mL pipet + pipette tips

Recipes

Dissociation media
50:50 Dispase and NCM, supplemented with 10% FBS, 1% PS, and 1% L-glutamine.
Seeding media
NCM supplemented with 10% FBS, 1% PS and 1% L-glutamine.

Coating

1. Thaw pre-aliquoted corning matrigel on ice until one ice crystal is left. Spray with 70% ethanol and transfer to laminar hood.
2. Immediately use the diluted matrigel solution to coat plates.
3. Incubate at 37°C with 5% CO2 for 1 hour, or overnight at 4°C.
4. If culture plate has been stored at 4°C, allow the plate to incubate at 37°C for at least 30 min before removing the matrigel solution.

Procedure

1. Transfer all tissue into a sterile 1.5 mL eppendorf tube containing the suspension media.
2. Incubate at 37°C with 5% CO2 for 20 minutes.
3. Aspirate excess media leaving the tissue in enough media so as not dry out, add 50 µL of dissociation media and triturate tissue 10 times with a 1000 µL pipette, avoid blowing air bubbles in the suspension.
4. Transfer cell suspension to a 15 mL Eppendorf tube and add suspension media up to 3 mL.
5. Centrifuge tissue at 200x g for 2 minutes.
6. Aspirate excess media and re-suspend the cell pellet in 100 mL fresh seeding media. Complete dissociation by triturating tissue with a 1000 mL pipet 10 times.
7. Divide the dissociated cells in two coated wells of an 8-well Ibidi chip.
2 wells: coated with matrigel
8. After 24 hours, aspirate half of the medium from each well and replace with fresh seeding media.

PROTOCOL 3

Materials and reagents

Dispase II (Gibco, Cat# 17105041)
NCM (Thermo Scientific, Cat# 88283)
FBS (Gibco, Cat# 16000044)
L-glutamine (Gibco, Cat# 25030081)
PS (Gibco, Cat# 15070063)
RI (STEMCELL Technologies, Cat# 72302)
Matrigel (Life sciences, Cat# A1413202)
Astrocytes (Gibco, Cat# N7745-100)

Equipment

1.5 mL Eppendorf tube
15 mL Eppendorf tube
Cell culture incubator
Centrifuge
8 well Ibidi chip
10-, 100- and 1000 mL pipet + pipette tips

Recipes

Dissociation media
50:50 Dispase and NCM, supplemented with 10% FBS, 1% PS, 1% L-glutamine, and 1:1000 RI.
Seeding media
NCM supplemented with 10% FBS, 1% PS, 1% L-glutamine and 1:1000 RI.

Coating

1. Thaw pre-aliquoted corning matrigel on ice until one ice crystal is left. Spray with 70% ethanol and transfer to laminar hood.
2. Immediately use the diluted matrigel solution to coat plates.
3. Incubate at 37°C with 5% CO2 for 1 hour, or overnight at 4°C.
4. If culture plate has been stored at 4°C, allow the plate to incubate at 37°C for at least 30 min before removing the matrigel solution.

***** To create a 3D culture mix matrigel and cell suspension 50:50 and coat directly on the well plate
******see protocol under product number

Procedure

1. Transfer all tissue into a sterile 1.5 mL eppendorf tube containing the suspension media.
2. Incubate at 37°C with 5% CO2 for 20 minutes.
3. Aspirate excess media leaving the tissue in enough media so as not dry out, add 50 µL of dissociation media and triturate tissue 10 times with a 1000 µL pipette, avoid blowing air bubbles in the suspension.
4. Transfer cell suspension to a 15 mL Eppendorf tube and add suspension media up to 3 mL.
5. Centrifuge tissue at 200x g for 2 minutes.
6. Aspirate excess media and re-suspend the cell pellet in 100 mL fresh seeding media. Complete dissociation by triturating tissue with a 1000 mL pipet 10 times.
7. Divide the dissociated cells in four coated wells of an 8-well Ibidi chip.
1 well: 3D culture with Matrigel+suspension*
1 well: Pre-seeded with astrocytes**
2 wells: coated with matrigel
8. After 24 hours, aspirate half of the medium from each well and replace with fresh seeding media.

PROTOCOL 4

Materials and reagents

Dispase II (Gibco, Cat# 17105041)
NCM (Thermo Scientific, Cat# 88283)
FBS (Gibco, Cat# 16000044)
PS (Gibco, Cat# 15070063)
L-glutamine (Gibco, Cat# 25030081)
RI (STEMCELL™ Technologies, Cat# 72302)
PLO (Sigma-Aldrich, Cat# P4957)
Laminin (Gibco, Cat# 23017015)
Fibronectin (Sigma-Aldrich, Cat# F4759)

Equipment

1.5 mL Eppendorf tube
15 mL Eppendorf tube
Cell culture incubator
Centrifuge
12 well-plate
10-, 100- and 1000 mL pipet + pipette tips
Cell counter (Thermo Fischer, Cat# AMQAX1000)

Recipes

Dissociation media
50:50 Dispase and NCM, supplemented with 10% FBS, 1% PS, 1% L-glutamine and 1:1000 RI.
Seeding media
NCM supplemented with 10% FBS, 1% PS, 1% L-glutamine and 1:1000 RI.
Cell count
50:50 Cell suspension and Trypsin blue.

Coating

Fibronectin
1. Dilute fibronectin in distilled water to make a 1-mg/mL stock solution
2. Add stock solution to make a working solution of 20 µg/mL
3. Add enough working solution to cover the surface of the culture vessel
4. Incubate the culture vessel at 37°C in with 5% CO2 for 1 hour.
5. Add enough working solution to cover the surface of the culture vessel.
6. Remove Fibronectin solution immediately before use, and fill the vessel with cell culture medium

PLO + laminin:
1. Coat well with PLO and allow vessel to sit at room temperature in laminar hood overnight.
2. Wash thoroughly with sterile water.
3. Coat with laminin and incubate at 37°C in with 5% CO2 for 2 hours.
4. Aspirate the laminin, add cell medium and incubate for 2 hours before seeding neurons.

Procedure

1. Transfer all tissue into a sterile 1.5 mL eppendorf tube containing the suspension media.
2. Incubate at 37°C with 5% CO2 for 20 minutes.
3. Aspirate excess media leaving the tissue in enough media so as not dry out, add 50 µL of dissociation media and triturate tissue 10 times with a 1000 µL pipette, avoid blowing air bubbles in the suspension.
4. Transfer cell suspension to a 15 mL Eppendorf tube and add suspension media up to 3 mL.
5. Centrifuge tissue at 200x g for 2 minutes.
6. Aspirate excess media and re-suspend the cell pellet in 100 mL fresh seeding media. Complete dissociation by triturating tissue with a 1000 mL pipet 10 times.
7. Perform a cell count
8. Divide the dissociated cells in four coated wells of an 8-well Ibidi chip.
2 wells: incubated 15 min before seeding
2 wells: PLO + laminin
2 wells: Fibronectin
9. After 24 hours, aspirate half of the medium from each well and replace with fresh seeding media.

Laminin solution
3 mL: L-15 medium
48 µL: Laminin
75 µL: Sodium bicarbonate

PROTOCOL 5

Materials and reagents

Dispase II (Gibco, Cat# 17105041)
NCM (Thermo Scientific, Cat# 88283)
FBS (Gibco, Cat# 16000044)
PS (Gibco, Cat# 15070063)
L-glutamine (Gibco, Cat# 25030081)
RI (STEMCELL™ Technologies, Cat# 72302)
PLO (Sigma-Aldrich, Cat# P4957)
Laminin (Gibco, Cat# 23017015)

Equipment

1.5 mL Eppendorf tube
Cell culture incubator
Centrifuge
8 well Ibidi chip
10-, 100- and 1000 mL pipet + pipette tips
Cell counter (Thermo Fischer, Cat# AMQAX1000)

Recipes

Dissociation media
50:50 Dispase and NCM, supplemented with 10% FBS, 1% PS, 1% L-glutamine, 1% PS, and 1:1000 RI.

Seeding media
NCM supplemented with 1% FBS and 1% PS.

Cell count
50:50 Cell suspension and Trypsin blue.

Coating

PLO + laminin:
1. Coat well with PLO and allow vessel to sit at room temperature in laminar hood overnight.
2. Wash thouroghly with sterile water.
3. Coat with laminin and incubate at 37°C in with 5% CO2 for 2 hours.
4. Aspirate the laminin, add cell medium and incubare for 2 hours before seeding neurons.

Laminin solution:
3 mL: L-15 medium
48 µL: Laminin
75 µL: Sodium bicarbonate

Procedure

1. Transfer all tissue into a sterile 1.5 mL eppendorf tube containing the suspension media.
2. Incubate at 37°C with 5% CO2 for 25 minutes.
3. Aspirate excess media leaving the tissue in enough media so as not dry out, add 50 µL of dissociation media and triturate tissue 10 times with a 1000 µL pipette, avoid blowing air bubbles in the suspension.
4. Transfer cell suspension to a 15 mL Eppendorf tube and add suspension media up to 3 mL.
5. Centrifuge tissue at 200x g for 2 minutes.
6. Aspirate excess media and re-suspend the cell pellet in 100 mL fresh seeding media. Complete dissociation by triturating tissue with a 1000 mL pipet 10 times.
7. Perform a cell count
8. Divide the dissociated cells in four coated wells of an 8-well Ibidi chip.
2 wells: PLO + laminin
9. After 24 hours, aspirate half of the medium from each well and replace with fresh seeding media.

PROTOCOL 6

Materials and reagents

Dispase II (Gibco, Cat# 17105041)
Neurobasal Plus Medium (Gibco, Cat# A3582901)
B27 (Gibco, Cat# A3653401)
GlutaMAX (Gibco, Cat# 35050061)
FBS (Gibco, Cat# 16000044)
RI (STEMCELL™ Technologies, Cat# 72302)
PDL (Gibco, Cat# A3890401)

Equipment

1.5 mL Eppendorf tube
Cell culture incubator
Centrifuge
12 well plate
10-, 100- and 1000 mL pipet + pipette tips
Cell counter (Thermo Fischer, Cat# AMQAX1000)

Recipes

Prepare complete Neurobasal Plus Medium:
See MAN0017319 by Gibco.

Dissociation media
50:50 Dispase and NPM, supplemented with 10% FBS and 1:1000 RI.

Seeding media
NPM supplemented with 10% FBS and 1:1000 RI.

Cell count
50:50 Cell suspension and Trypsin blue.

Coating

1. Dilute the PDL solution in sterile DPBS to prepare a 50 µg/mL working solution (50:50).
2. Coat the surface of the culture vessel with the working solution of PDL.
3. Incubate the culture vessel at room temperature for 1 hour.
4. Remove the PDL solution and rinse culture surface 3 times with sterile distilled water. Make sure to rinse the culture vessel thoroughly as excess Poly-D-Lysine solution can be toxic to the cells.
5. Remove distilled water and leave the coated culture vessel uncovered in the laminar hood to dry. The culture surface should be fully dry after 2 hours. Plates can be used immediately once dry or can be stored dry at 4°C, tightly wrap the vessel with Parafilm film and use within one week of coating.

Procedure

1. Transfer all tissue into a sterile 1.5 mL eppendorf tube containing the suspension media.
2. Incubate at 37°C with 5% CO2 for 30 minutes.
3. Aspirate excess media leaving the tissue in enough media so as not dry out, add 50 µL of dissociation media and triturate tissue 10 times with a 1000 µL pipette, avoid blowing air bubbles in the suspension.
4. Transfer cell suspension to a 15 mL Eppendorf tube and add suspension media up to 3 mL.
5. Centrifuge tissue at 200x g for 2 minutes.
6. Aspirate excess media and re-suspend the cell pellet in 100 mL fresh seeding media. Complete dissociation by triturating tissue with a 1000 mL pipet 10 times.
7. Perform a cell count
8. Divide the dissociated cells in two coated wells of a 12 well plate.
2 wells: PDL
9. After 24 hours, aspirate half of the medium from each well and replace with fresh seeding media.

PROTOCOL 7

Materials and reagents

Dispase II (Gibco, Cat# 17105041)
NPM (Gibco, Cat# A3582901)
B27 (Gibco, Cat# A3653401)
GlutaMAX (Gibco, Cat# 35050061)
FBS (Gibco, Cat# 16000044)
DNase I
RI (STEMCELL™ Technologies, Cat# 72302)
PDL (Gibco, Cat# A3890401)

Equipment

1.5 mL Eppendorf tube
Cell culture incubator
Centrifuge
12 well plate
10-, 100- and 1000 mL pipet + pipette tips
Cell counter (Thermo Fischer, Cat# AMQAX1000)

Recipes

Prepare complete Neurobasal Plus Medium:
See MAN0017319 by Gibco.

Dissociation media
50:50 Dispase and NPM, supplemented with 10% FBS, 1:1000 RI and 1% DNase I.

Seeding media
NPM supplemented with 10% FBS, 1:1000 RI, and 1% DNase I.

Cell count
50:50 Cell suspension and Trypsin blue.

Coating

1. Dilute the PDL solution in sterile DPBS to prepare a 50 µg/mL working solution (50:50).
2. Coat the surface of the culture vessel with the working solution of PDL.
3. Incubate the culture vessel at room temperature for 1 hour.
4. Remove the PDL solution and rinse culture surface 3 times with sterile distilled water. Make sure to rinse the culture vessel thoroughly as excess Poly-D-Lysine solution can be toxic to the cells.
5. Remove distilled water and leave the coated culture vessel uncovered in the laminar hood to dry. The culture surface should be fully dry after 2 hours. Plates can be used immediately once dry or can be stored dry at 4°C, tightly wrap the vessel with Parafilm film and use within one week of coating.

Procedure

1. Transfer all tissue into a sterile 1.5 mL eppendorf tube containing the suspension media.
2. Incubate at 37°C with 5% CO2 for 30 minutes.
3. Aspirate excess media leaving the tissue in enough media so as not dry out, add 50 µL of dissociation media and triturate tissue 10 times with a 1000 µL pipette, avoid blowing air bubbles in the suspension.
4. Transfer cell suspension to a 15 mL Eppendorf tube and add suspension media up to 3 mL.
5. Centrifuge tissue at 200x g for 2 minutes.
6. Aspirate excess media and re-suspend the cell pellet in 100 mL fresh seeding media. Complete dissociation by triturating tissue with a 1000 mL pipet 10 times.
7. Perform a cell count
8. Divide the dissociated cells in two coated wells of a 12 well plate.
2 wells: PDL
9. After 24 hours, aspirate half of the medium from each well and replace with fresh seeding media.

PROTOCOL 8

Materials and reagents

Dispase II (Gibco, Cat# 17105041)
NPM (Gibco, Cat# A3582901)
B27 (Gibco, Cat# A3653401)
GlutaMAX (Gibco, Cat# 35050061)
FBS (Gibco, Cat# 16000044)
RI (STEMCELL™ Technologies, Cat# 72302)
PDL (Gibco, Cat# A3890401)

Equipment

1.5 mL Eppendorf tube
Cell culture incubator
Centrifuge
8 well Ibidi chip
10-, 100- and 1000 mL pipet + pipette tips

Recipes

Prepare complete Neurobasal Plus Medium:
See MAN0017319 by Gibco.

Dissociation media
50:50 Dispase and NPM, supplemented with 10% FBS, 1:1000 RI and 1% DNase I.

Seeding media
NPM supplemented with 10% FBS and 1:1000 RI.

Cell count
50:50 Cell suspension and Trypsin blue.

Coating

1. Dilute the PDL solution in sterile DPBS to prepare a 50 µg/mL working solution (50:50).
2. Coat the surface of the culture vessel with the working solution of PDL.
3. Incubate the culture vessel at room temperature for 1 hour.
4. Remove the PDL solution and rinse culture surface 3 times with sterile distilled water. Make sure to rinse the culture vessel thoroughly as excess Poly-D-Lysine solution can be toxic to the cells.
5. Remove distilled water and leave the coated culture vessel uncovered in the laminar hood to dry. The culture surface should be fully dry after 2 hours. Plates can be used immediately once dry or can be stored dry at 4°C, tightly wrap the vessel with Parafilm film and use within one week of coating.

Procedure

1. Transfer all tissue into a sterile 1.5 mL eppendorf tube containing the suspension media.
2. Incubate at 37°C with 5% CO2 for 30 minutes.
3. Aspirate excess media leaving the tissue in enough media so as not dry out, add 50 µL of dissociation media and triturate tissue 10 times with a 1000 µL pipette, avoid blowing air bubbles in the suspension.
4. Transfer cell suspension to a 15 mL Eppendorf tube and add suspension media up to 3 mL.
5. Centrifuge tissue at 200x g for 2 minutes.
6. Aspirate excess media and re-suspend the cell pellet in 100 mL fresh seeding media. Complete dissociation by triturating tissue with a 1000 mL pipet 10 times.
7. Perform a cell count
8. Divide the dissociated cells in one coated wells of a 8 well Ibidi chip.
2 wells: PDL
9. After 24 hours, aspirate half of the medium from each well and replace with fresh seeding media.

PROTOCOL 9

Materials and reagents

Dispase II (Gibco, Cat# 17105041)
NPM (Gibco, Cat# A3582901)
B27 (Gibco, Cat# A3653401)
GlutaMAX (Gibco, Cat# 35050061)
FBS (Gibco, Cat# 16000044)
RI (STEMCELL™ Technologies, Cat# 72302)
Geltrex

Equipment

1.5 mL Eppendorf tube
Cell culture incubator
Centrifuge
8 well Ibidi chip
10-, 100- and 1000 mL pipet + pipette tips
Cell counter (Thermo Fischer, Cat# AMQAX1000)

Recipes

Prepare complete Neurobasal Plus Medium:
See MAN0017319 by Gibco.

Dissociation media
50:50 Dispase and NPM, supplemented with 10% FBS and 1:1000 RI.

Seeding media
NPM supplemented with 10% FBS and 1:1000 RI.

Cell count
50:50 Cell suspension and Trypsin blue.

Coating

1. Dilute the PDL solution in sterile DPBS to prepare a 50 µg/mL working solution (50:50).
2. Coat the surface of the culture vessel with the working solution of PDL.
3. Incubate the culture vessel at room temperature for 1 hour.
4. Remove the PDL solution and rinse culture surface 3 times with sterile distilled water. Make sure to rinse the culture vessel thoroughly as excess Poly-D-Lysine solution can be toxic to the cells.
5. Remove distilled water and leave the coated culture vessel uncovered in the laminar hood to dry. The culture surface should be fully dry after 2 hours. Plates can be used immediately once dry or can be stored dry at 4°C, tightly wrap the vessel with Parafilm film and use within one week of coating.

Procedure

1. Transfer all tissue into a sterile 1.5 mL eppendorf tube containing the suspension media.
2. Incubate at 37°C with 5% CO2 for 30 minutes.
3. Aspirate excess media leaving the tissue in enough media so as not dry out, add 50 µL of dissociation media and triturate tissue 10 times with a 1000 µL pipette, avoid blowing air bubbles in the suspension.
4. Transfer cell suspension to a 15 mL Eppendorf tube and add suspension media up to 3 mL.
5. Centrifuge tissue at 200x g for 2 minutes.
6. Aspirate excess media and re-suspend the cell pellet in 100 mL fresh seeding media. Complete dissociation by triturating tissue with a 1000 mL pipet 10 times.
7. Perform a cell count
8. Divide the dissociated cells in two coated wells of a 8 well Ibidi chip.
2 wells: Geltrex
9. After 24 hours, aspirate half of the medium from each well and replace with fresh seeding media.

PROTOCOL 10

Materials and reagents

Dispase II (Gibco, Cat# 17105041)
NPM (Gibco, Cat# A3582901)
B27 (Gibco, Cat# A3653401)
GlutaMAX (Gibco, Cat# 35050061)
FBS (Gibco, Cat# 16000044)
RI (STEMCELL™ Technologies, Cat# 72302)
PDL (Gibco, Cat# A3890401)
PS (Gibco, Cat# 15070063)

Equipment

1.5 mL Eppendorf tube
Cell culture incubator
Centrifuge
96 well plate
10-, 100- and 1000 mL pipet + pipette tips
Cell counter (Thermo Fischer, Cat# AMQAX1000)

Recipes

Prepare complete Neurobasal Plus Medium:
See MAN0017319 by Gibco.

Dissociation media
50:50 Dispase and NPM, supplemented with 10% FBS, 1:1000 RI and 1% DNase I.

Seeding media
NPM supplemented with 10% FBS, 1:1000 RI, and 0.5% PS.

Cell count
50:50 Cell suspension and Trypsin blue.

Coating

1. Dilute the PDL solution in sterile DPBS to prepare a 50 µg/mL working solution (50:50).
2. Coat the surface of the culture vessel with the working solution of PDL.
3. Incubate the culture vessel at room temperature for 1 hour.
4. Remove the PDL solution and rinse culture surface 3 times with sterile distilled water. Make sure to rinse the culture vessel thoroughly as excess Poly-D-Lysine solution can be toxic to the cells.
5. Remove distilled water and leave the coated culture vessel uncovered in the laminar hood to dry. The culture surface should be fully dry after 2 hours. Plates can be used immediately once dry or can be stored dry at 4°C, tightly wrap the vessel with Parafilm film and use within one week of coating.

Procedure

1. Transfer all tissue into a sterile 1.5 mL eppendorf tube containing the suspension media.
2. Incubate at 37°C with 5% CO2 for 30 minutes.
3. Aspirate excess media leaving the tissue in enough media so as not dry out, add 50 µL of dissociation media and triturate tissue 10 times with a 1000 µL pipette, avoid blowing air bubbles in the suspension.
4. Transfer cell suspension to a 15 mL Eppendorf tube and add suspension media up to 3 mL.
5. Centrifuge tissue at 200x g for 2 minutes.
6. Aspirate excess media and re-suspend the cell pellet in 100 mL fresh seeding media. Complete dissociation by triturating tissue with a 1000 mL pipet 10 times.
7. Perform a cell count
8. Divide the dissociated cells in one coated wells of 96 well plate
1 well: PDL
9. After 24 hours, aspirate half of the medium from each well and replace with fresh seeding media.

PROTOCOL 11

Materials and reagents

Dispase II (Gibco, Cat# 17105041)
NPM (Gibco, Cat# A3582901)
B27 (Gibco, Cat# A3653401)
GlutaMAX (Gibco, Cat# 35050061)
FBS (Gibco, Cat# 16000044)
RI (STEMCELL™ Technologies, Cat# 72302)
PLO (Sigma-Aldrich, Cat# P4957)
Laminin (Gibco, Cat# 23017015)
Astrocytes (Gibco, Cat# N7745-100)

Equipment

1.5 mL Eppendorf tube
Cell culture incubator
Centrifuge
Microfluidic chip
10-, 100- and 1000 mL pipet + pipette tips
Cell counter (Thermo Fischer, Cat# AMQAX1000)

Recipes

Prepare complete Neurobasal Plus Medium:
See MAN0017319 by Gibco.

Dissociation media
50:50 Dispase and NPM, supplemented with 10% FBS, 1:1000 RI and 1% DNase I.

Seeding media
NPM supplemented with 10% FBS, 1:1000 RI, and 0.5% PS.

Cell count
50:50 Cell suspension and Trypsin blue.

Cleaning and sterilization of microfluidic chips:
See appendix 2.

Coating

PLO + laminin:
1. Coat well with PLO and allow vessel to sit at room temperature in laminar hood overnight.
2. Wash thoroughly with sterile water.
3. Coat with laminin and incubate at 37°C in with 5% CO2 for 2 hours.
4. Aspirate the laminin, add cell medium and incubate for 2 hours before seeding neurons.

Laminin solution:
3 mL: L-15 medium
48 µL: Laminin
75 µL: Sodium bicarbonate

**Astrocytes: see protocol under product number

Procedure

1. Transfer all tissue into a sterile 1.5 mL eppendorf tube containing the suspension media.
2. Incubate at 37°C with 5% CO2 for 30 minutes.
3. Aspirate excess media leaving the tissue in enough media so as not dry out, add 50 µL of dissociation media and triturate tissue 10 times with a 1000 µL pipette, avoid blowing air bubbles in the suspension.
4. Transfer cell suspension to a 15 mL Eppendorf tube and add suspension media up to 3 mL.
5. Centrifuge tissue at 200x g for 2 minutes.
6. Aspirate excess media and re-suspend the cell pellet in 100 mL fresh seeding media. Complete dissociation by triturating tissue with a 1000 mL pipet 10 times.
7. Perform a cell count
8. Divide the dissociated cells in six coated wells of two microfluidic chips*
6 wells: PLO + laminin + Astrocytes**
9. After 24 hours, aspirate half of the medium from each well and replace with fresh seeding media.

*the middle well of each microfluidic chip was seeded with CA3 pyramidal neurons, while the two outermost wells of each microfluidic chip was seeded with EC layer II neurons.

PROTOCOL 12

Materials and reagents

Dispase II (Gibco, Cat# 17105041)
NPM (Gibco, Cat# A3582901)
B27 (Gibco, Cat# A3653401)
GlutaMAX (Gibco, Cat# 35050061)
FBS (Gibco, Cat# 16000044)
RI (STEMCELL™ Technologies, Cat# 72302)
PDL (Gibco, Cat# A3890401)
Astrocytes (Gibco, Cat# N7745-100)

Equipment

1.5 mL Eppendorf tube
Cell culture incubator
Centrifuge
96 well plate
10-, 100- and 1000 mL pipet + pipette tips
Cell counter (Thermo Fischer, Cat# AMQAX1000)

Recipes

Prepare complete Neurobasal Plus Medium:
See MAN0017319 by Gibco.

Dissociation media
50:50 Dispase and NPM, supplemented with 10% FBS, 1:1000 RI and 1% DNase I.

Seeding media
NPM supplemented with 10% FBS, 1:1000 RI, and 0.5% PS.

Cell count
50:50 Cell suspension and Trypsin blue.

Coating

1. Dilute the PDL solution in sterile DPBS to prepare a 50 µg/mL working solution (50:50).
2. Coat the surface of the culture vessel with the working solution of PDL.
3. Incubate the culture vessel at room temperature for 1 hour.
4. Remove the PDL solution and rinse culture surface 3 times with sterile distilled water. Make sure to rinse the culture vessel thoroughly as excess Poly-D-Lysine solution can be toxic to the cells.
5. Remove distilled water and leave the coated culture vessel uncovered in the laminar hood to dry. The culture surface should be fully dry after 2 hours. Plates can be used immediately once dry or can be stored dry at 4°C, tightly wrap the vessel with Parafilm film and use within one week of coating.

Procedure

1. Transfer all tissue into a sterile 1.5 mL eppendorf tube containing the suspension media.
2. Incubate at 37°C with 5% CO2 for 30 minutes.
3. Aspirate excess media leaving the tissue in enough media so as not dry out, add 50 µL of dissociation media and triturate tissue 10 times with a 1000 µL pipette, avoid blowing air bubbles in the suspension.
4. Transfer cell suspension to a 15 mL Eppendorf tube and add suspension media up to 3 mL.
5. Centrifuge tissue at 200x g for 2 minutes.
6. Aspirate excess media and re-suspend the cell pellet in 100 mL fresh seeding media. Complete dissociation by triturating tissue with a 1000 mL pipet 10 times.
7. Perform a cell count
8. Divide the dissociated cells in two coated wells of the 96 well plate
6 wells: PDL + Astrocytes
9. After 24 hours, aspirate half of the medium from each well and replace with fresh seeding media.

PROTOCOL 13

Materials and reagents

Dispase II (Gibco, Cat# 17105041)
NPM (Gibco, Cat# A3582901)
B27 (Gibco, Cat# A3653401)
GlutaMAX (Gibco, Cat# 35050061)
FBS (Gibco, Cat# 16000044)
RI (STEMCELL™ Technologies, Cat# 72302)
PLO (Sigma-Aldrich, Cat# P4957)
Laminin (Gibco, Cat# 23017015)
Astrocytes (Gibco, Cat# N7745-100)

Equipment

1.5 mL Eppendorf tube
Cell culture incubator
Centrifuge
6 well MEA
10-, 100- and 1000 mL pipet + pipette tips
Cell counter (Thermo Fischer, Cat# AMQAX1000)

Recipes

Prepare complete Neurobasal Plus Medium:
See MAN0017319 by Gibco.

Dissociation media
50:50 Dispase and NPM, supplemented with 10% FBS, 1:1000 RI and 1% DNase I.

Seeding media
NPM supplemented with 10% FBS, 1:1000 RI, and 0.5% PS.

Cell count
50:50 Cell suspension and Trypsin blue.

Cleaning and sterilization of microfluidic chips:
See appendix 2.

Coating

PLO + laminin:
1. Coat well with PLO and allow vessel to sit at room temperature in laminar hood overnight.
2. Wash thoroughly with sterile water.
3. Coat with laminin and incubate at 37°C in with 5% CO2 for 2 hours.
4. Aspirate the laminin, add cell medium and incubate for 2 hours before seeding neurons.

Laminin solution:
3 mL: L-15 medium
48 µL: Laminin
75 µL: Sodium bicarbonate

*Astrocytes: see protocol under product number

Procedure

1. Transfer all tissue into a sterile 1.5 mL eppendorf tube containing the suspension media.
2. Incubate at 37°C with 5% CO2 for 30 minutes.
3. Aspirate excess media leaving the tissue in enough media so as not dry out, add 50 µL of dissociation media and triturate tissue 10 times with a 1000 µL pipette, avoid blowing air bubbles in the suspension.
4. Transfer cell suspension to a 15 mL Eppendorf tube and add suspension media up to 3 mL.
5. Centrifuge tissue at 200x g for 2 minutes.
6. Aspirate excess media and re-suspend the cell pellet in 100 mL fresh seeding media. Complete dissociation by triturating tissue with a 1000 mL pipet 10 times.
7. Perform a cell count
8. Divide the dissociated cells in four coated wells of 6 well MEAs*
6 wells: PLO + laminin + Astrocytes*
9. After 24 hours, aspirate half of the medium from each well and replace with fresh seeding media.

*three of the wells was seeded with EC layer II neurons, while one well with CA3 pyramidal neurons

PROTOCOL 14

Materials and reagents

Dispase II (Gibco, Cat# 17105041)
NPM (Gibco, Cat# A3582901)
B27 (Gibco, Cat# A3653401)
GlutaMAX (Gibco, Cat# 35050061)
FBS (Gibco, Cat# 16000044)
RI (STEMCELL™ Technologies, Cat# 72302)
PDL (Gibco, Cat# A3890401)

Equipment

1.5 mL Eppendorf tube
Cell culture incubator
Centrifuge
12 well plate
10-, 100- and 1000 mL pipet + pipette tips
Cell counter (Thermo Fischer, Cat# AMQAX1000)

Recipes

Prepare complete Neurobasal Plus Medium:
See MAN0017319 by Gibco.

Dissociation media
50:50 Dispase and NPM, supplemented with 10% FBS and 1:1000 RI.

Seeding media
NPM supplemented with 10% FBS and 1:1000 RI.

Cell count
50:50 Cell suspension and Trypsin blue.

Coating

1. Dilute the PDL solution in sterile DPBS to prepare a 50 µg/mL working solution (50:50).
2. Coat the surface of the culture vessel with the working solution of PDL.
3. Incubate the culture vessel at room temperature for 1 hour.
4. Remove the PDL solution and rinse culture surface 3 times with sterile distilled water. Make sure to rinse the culture vessel thoroughly as excess Poly-D-Lysine solution can be toxic to the cells.
5. Remove distilled water and leave the coated culture vessel uncovered in the laminar hood to dry. The culture surface should be fully dry after 2 hours. Plates can be used immediately once dry or can be stored dry at 4°C, tightly wrap the vessel with Parafilm film and use within one week of coating.

Procedure

1. Transfer all tissue into a sterile 1.5 mL eppendorf tube containing the suspension media.
2. Incubate at 37°C with 5% CO2 for 30 minutes.
3. Aspirate excess media leaving the tissue in enough media so as not dry out, add 50 µL of dissociation media and triturate tissue 10 times with a 1000 µL pipette, avoid blowing air bubbles in the suspension.
4. Transfer cell suspension to a 15 mL Eppendorf tube and add suspension media up to 3 mL.
5. Centrifuge tissue at 200x g for 2 minutes.
6. Aspirate excess media and re-suspend the cell pellet in 100 mL fresh seeding media. Complete dissociation by triturating tissue with a 1000 mL pipet 10 times.
7. Perform a cell count
8. Divide the dissociated cells in two coated wells of a 12 well plate.
2 wells: PDL
9. After 24 hours, aspirate half of the medium from each well and replace with fresh seeding media.

APPENDIX 2 - SEX, AGE AND GENOTYPE OF ALL ANIMALS IN EACH EXPERIMENT

Table 4. List of sex, age, genotype of all animals in each experiment.

Experiment	Sex	Age	Genotype	
1	F	P36	APP/PS1 +/- x Ck2/tTa* +/-	**AD**
2	M	P43	APP/PS1 **+/-** x Ck2/tTa +/-	**AD**
3	M	P28	APP/PS1 -/- x Ck2/tTa -/-	**Control**
	M	P28	APP/PS1 -/- x Ck2/tTa -/-	**Control**
4	F	P29	APP/PS1 +/- x Ck2/tTa -/-	**AD**
	F	P29	APP/PS1 +/- x Ck2/tTa +/-	**AD**
5	M	P36	APP/PS1 -/- x Ck2/tTa -/-	**Control**
	M	P36	APP/PS1 -/- x Ck2/tTa -/-	**Control**
6	M	P38	APP/PS1 +/- x Ck2/tTa -/-	**AD**
7	F	P59	APP/PS1 +/- x Ck2/tTa -/-	**AD**
8	F	P65	APP/PS1 +/- x Ck2/tTa -/-	**AD**
	F	P65	APP/PS1 +/- x Ck2/tTa +/-	**AD**
9	F	P54	APP/PS1 +/- x Ck2/tTa ?	**AD**
10	F	P62	APP/PS1 -/- x Ck2/tTa -/-	**Control**
	F	P62	APP/PS1 -/- x Ck2/tTa +/-	**Control**
11	M	P75	APP/PS1 +/- x Ck2/tTa -/-	**AD**
12	M	P75	APP/PS1 +/- Ck2/tTa -/-	**AD**
13	M	P87	APP/PS1 +/- Ck2/tTa +/-	**AD**
14	M	P88	APP/PS1 -/- x Ck2/tTa -/-	**Control**
	M	P88	APP/PS1 -/- x Ck2/tTa -/-	**Control**

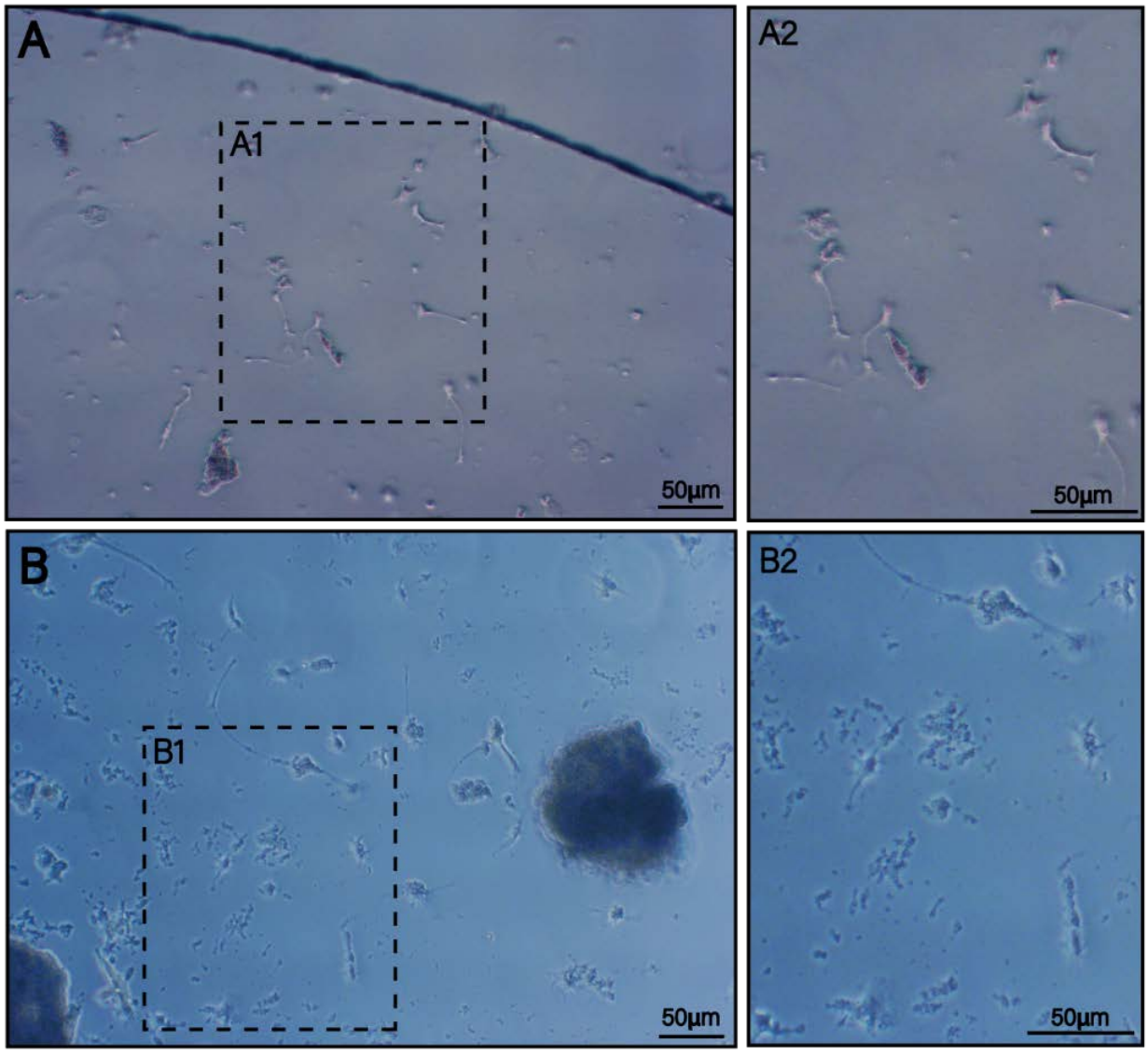
*Ck2/tTa, also called CamKII-tTa, is a calcium/calmodulin-dependent protein kinase II alpha transgene. The mouse model included in this thesis were also gene-expressed for CamKII-tTa, in addition to the APP/PS1 mutations. In this thesis, all animals positively expressed for APP/PS1 (+/-) was included as AD-phenotype, while animals negatively expressed for APP/PS1 (-/-) was used as controls.

APPENDIX 3 - RESULTS FROM INITIAL NEURONAL SURVIVAL

Table 5. Initial neuronal survival calculated as a mean (%) from cell count A and B.

Experiment	Initial neuronal survival
1	Did not do a cell count.
2	Did not do a cell count.
3	Did not do a cell count.
4	**A:** 37% **B:** 15% ~26%
5	**A:** 51% **B:** 65% ~58%
6	**A:** 17% **B:** 54% ~35 %
7	**A:** 40% **B:** 38% ~39 %
8	Did not do a cell count.
9	**A:** 34% **B:** 51% ~42%
10	**A:** 15% **B:** 48% ~31%
11	**A:** 0% **B:** 25% ~25%
12	**A:** 0% **B:** 25% ~25%
13	**A**: 27% **B:** 14% ~20%
14	**A:** 15% **B:** 44% ~29%

SUPPLEMENTARY FIGURE 1.



Supplementary figure 1. More debris with DNase I included in the protocol. Both experiments was performed with the same protocol, with exception from adding DNase I in experiment 7 (B). More debris was observed in experiment 7 (B) compared to in experiment 6 (A). **(A)** Image is from experiment 6 and taken on microscope with 10x objective, eight days after plating of neurons. B2 is an enlarged inset from B1. **(B)** Image is from experiment 7 and taken on microscope with 10x objective, eight days after plating of neurons. B2 is an enlarged inset from B1. All scale bars as indicated.