The development and optimization of an in vitro E18 rat cortex epilepsy model

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Abstract

This study describes efforts to create a cell culture-based in vitro model of epilepsy, based on E18 rat cortex material. To study this model, two assays are used. The calcium-6 assay is used to measure network activity in vitro. Immunofluorescent staining is performed against a variety of cell types commonly found in the central nervous system and the cortex in efforts to characterize and quantify cells in culture. This report describes comparisons and choices made for cell seeding density, medium change protocols, culture plate coatings, medium choices, the effects of medium changes on network development, and long-term culture as part of optimization of the culturing conditions. Additionally, it compares epilepsy models commonly used in vitro including addition of convulsant drugs like 4-Aminopyridine as well as incubation of cells in low magnesium conditions. The final part of the report describes preliminary efforts to determine the effects of nutritional supplementation and ketosis inducing conditions in culture.

Layman's summary

Epilepsy is a term used to describe disorders where the electrical signalling between nerve cells in the brain is strongly altered, characterized by uncontrolled signal transduction. In a sizable (30%) part of patients with epilepsy the standard treatment of these disorders with pharmaceuticals aimed at controlling the altered brain activity becomes ineffective due to acquired immunity against the drugs. For these patients, neurosurgery and dietary intervention are commonly explored options to alleviate symptoms of epilepsy.

The ketogenic diet is a diet rich in lipids over traditionally carbohydrate-rich diets. It was developed to treat epilepsy back in the 1920s. It's functionality in treating epilepsy is not fully understood but the altered neural activity is thought to rely on changing energy metabolism associated with the swap from glucose and carbohydrate metabolism to metabolism relying on ketone body formation.

This report describes the development of a cortex-based cell culture model of epilepsy which can be used to study cells under conditions like a ketogenic diet and the impact of nutritional interventions on culture development and nerve cell activity. The cells used for this model are directly taken from embryonic rat cortex material and include all relevant cells normally found in the cortex at this stage of embryonic development.

These cells are grown for 2-3 weeks and then subjected to either of 2 different assays. The calcium-6 assay gives a measure of network activity of the nerve cells in culture. Specifically, cultures of cortical cells are known to develop <1Hz oscillations where nerve cells depolarize as a network. These network oscillations can be recorded, and the data can be used as a measure of activity and network development. Immunofluorescent staining is used to quantify major cell types of the cortex like neurons, astrocytes, and oligodendrocytes, and characterize cell sub types and marker expression in the cultures. This collective of data is used to explain differences seen in the results of the calcium-6 assay.

The study consists of three major parts. The first part describes efforts to create and optimize a culture method for the cortical cells to grow a healthy and active neuronal network capable of signal transduction. This includes culture duration, seeding density of the cells, media compositions, medium changes and more. The second part of the report describes the choice for a model of epilepsy. A comparison between two different methods of inducing epileptic symptoms in the culture is described. Finally, the third part of the report describes preliminary efforts to determine if the calcium-6 assay and the model are sensitive enough to pick up small changes in network development and behaviour related to nutritional intervention and altered dietary conditions.

In conclusion, we report to have created a concept (in development) for a cell culture model of epilepsy. The model is sensitive enough to pick up effects of nutritional intervention on network development and activity. The induction of epilepsy presents with increased frequency and synchronized network activity similar to findings described in literature and traditional descriptions of epileptic behaviour. Most culturing conditions are optimized, although some work is still necessary to determine optimal medium composition. Major cell types in the cultures are found in ratios comparable to literature for most conditions, although medium composition strongly affects this. Microglia are not found in the culture, and future adaptations to the model need to adjust for the loss of these cells in culture. More research is necessary to optimize the culture conditions and determine the value of the model to study nutritional intervention.

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Keywords and abbreviations

AMPAR - α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor is a receptor for glutamate in the Central nervous system.

B – Component B recording buffer from the calcium-6 assay kit. Based on HBSS +/+ with 20 mM HEPES buffer.

BHB – Sodium-3-beta hydroxybutyrate, a ketone body involved in cellular energy provision in a ketogenic diet. An alternative to D-glucose.

BP – BrainPhys medium, a specialty medium developed by Bardy. Based on composition of cerebrospinal fluid and used to enhance network activity of neurons in culture.

Calcium-6 assay – Functional assay developed by Molecular Devices allowing for quantitative measurements of calcium movement between cytoplasm of cells and extracellular volumes as measured by fluorescent signal.

CNS – Central nervous system, including the brain and spinal cord.

CSF – Cerebrospinal fluid.

DIV – Days In Vitro, refers to culture durations.

DMEM – Dulbecco's modified eagles' medium.

GABAR - γ-Aminobutyric acid receptor, a common GABA receptor.

GFAP – Glial Fibrillary Acidic Protein, marker expressed by Astrocytes and upregulated upon activation.

Iba-1 – Allograft inflammatory factor 1 (AIF-1), a marker exclusive to macrophages and microglia. Upregulated when those cells are activated.

LAM – Natural Mouse Laminin, refers to the coating with PDL and Laminin.

LMg – Low Magnesium, refers to a version of HBSS -/- with extra Calcium chloride added to raise it to levels of HBSS +/+, but keeping low levels of Magnesium.

MYE – Myelination medium change protocol, wherein medium is supplemented at the DIV 3 timepoint and then left untouched until the calcium-6 assay or fixation.

MBP – Myelin basic protein. The main protein product created by Oligodendrocytes for myelin sheath formation.

MYM – Myelination medium. Developed by Lariosa. Enhances development/maturation of oligodendrocytes.

NB – Neurobasal medium.

NB-A – Neurobasal medium without D-glucose.

NC – NeuroCult neuronal plating medium. Medium developed by Stem cell technologies. Seeding medium commonly used in addition to BP medium.

NeuN - Fox-3, Rbfox3, or Hexaribonucleotide Binding Protein-3. A nuclear marker exclusive for post-mitotic mature neurons.

NI – Nutritional intervention, a nutrient mixture patented by Danone/Nutricia research institute.

NMDAR - N-methyl-D-aspartate receptor, a common glutamate receptor in the central nervous system.

Olig2 – Oligodendrocyte transcription factor 2, a specific lineage marker for oligodendrocytes located in the nucleus.

OSC – Oscillation medium change protocol, wherein medium is (partially) changed every 3-4 days.

PDL – Poly-D-Lysine, refers to the substance and the coating with Poly-D-Lysine. Coating material commonly used for neuronal cultures

P/S – Penicillin/Streptomycin, a mixture of two culturing antibiotics.

SD – Standard deviation. Indicates a measure of spread in resulting values of an experimental group.

Sox9 – A transcription factor expressed by a large part of the cells in the developing central nervous system, can be used to label astrocytes.

4-AP – 4-Aminopyridine, a general convulsant used to induce epileptic behaviour in neuronal cultures.

+/+, -/-, HBSS – Hank's Buffered Saline Solution, a salt solution used for diluting substances and washing cells. A +/+ version includes Magnesium and Calcium, and a -/- version does not contain those minerals.

Introduction

Epilepsy refers to a collection of disorders of the central nervous system characterized by progressively worsening-, and recurring seizures. It is the fourth most common neurological disorder in the world by incidence, with an estimated 50 million people worldwide suffering from this debilitating disorder (WHO, 2023). Patients suffering from forms of epilepsy are at risk of physical and mental injuries, either directly or indirectly (due to for example loss of motor control or awareness of their surroundings) because of the seizures they experience. The underlying cause of epilepsy relates to excessive and/or abnormal neuronal activity in the central nervous system. Whilst the cause of most forms of epilepsy is unknown and recurring seizures seem to have no underlying direct cause (Beghi et al., 2015), onset is often linked to prior brain injuries including trauma, stroke, (previous) presence of a brain tumour, infections of the central nervous system, and birth deficits (Beghi et al., 2015; WHO, 2023). A small subset of cases is linked to genetics as well (Pandolfo, 2011). In around 70% of cases, epilepsy can be controlled with anti-epileptic medication (D'Andrea Meira et al., 2019). In the remaining cases, those where drug-resistant epilepsy makes treatment with drugs ineffective, other means including surgery, neurostimulation and dietary changes are used to treat the disorders (Chang & Lowenstein, 2003).

The ketogenic diet (Keto diet, KD), a diet consisting of a high lipid-to-carbohydrate ratio, has traditionally been used since the 1920s to try and treat resistant cases of Epilepsy (Calderón et al., 2017; D'Andrea Meira et al., 2019; García-Rodríguez & Giménez-Cassina, 2021; Guzmán & Blázquez, 2004; Ji et al., 2020; Lutas & Yellen, 2013; Rho, 2017). The keto diet is generally thought to mimic a state of fasting, whereby the lack of carbohydrates causes the metabolism of the consumer to switch to the use of fats (fatty acids specifically) as a primary energy source (D'Andrea Meira et al., 2019; García-Rodríguez & Giménez-Cassina, 2021). Catabolism of fatty acids in the liver produces so-called ketone bodies (KBs), which are used as an alternative source for ATP production in the body (García-Rodríguez & Giménez-Cassina, 2021; Guzmán & Blázquez, 2004). Interestingly, supporting cells in the brain, glial cells, are also capable of producing ketone bodies separately from the liver (Silva et al., 2022). These glia-derived ketone bodies can supplement other cells of the central nervous system with energy, although low levels of glucose are still necessary for proper homeostasis in the brain (Silva et al., 2022). Whilst the method through which the keto diet affects epilepsy is poorly understood, it is currently believed that the diet affects multiple mechanisms responsible for biochemical alterations, including cellular substrates and mediators involved in neuronal hyperexcitability (García-Rodríguez & Giménez-Cassina, 2021). Metabolic changes in blood and cerebrospinal fluid including lowered glucose levels and increased ketone body levels are linked to seizure reduction (Lutas & Yellen, 2013). Additionally, it has been proposed that altered mitochondria functioning and energy reserves play a role in the KD mechanisms, which could result in synapse stabilization and a decrease in neuroexcitation (D'Andrea Meira et al., 2019). Ketone bodies have also been linked to anti-convulsant effects in a variety of animal models and injections with ketone bodies let to reduction of seizure susceptibility (D'Andrea Meira et al., 2019). Ketone bodies have also been found to exert direct inhibitory effects on vesicular glutamate transporter (VGluT) activity (D'Andrea Meira et al., 2019). One of the main shortcomings of the keto diet is its rigid format, and unpalatable and restrictive nature. This leads to low percentages of patients maintaining the diet for extended periods of time (D'Andrea Meira et al., 2019; Lutas & Yellen, 2013; Rho, 2017). To try and combat this low patient compliance, new variants of the keto diet are being researched that have a lower lipid-to-carbohydrate ratio and that are easier to adhere to. Diets like the modified Atkins diet (MAD), a diet with high fat content but also more protein and less restrictive of calorie intake, has been tested and has shown similar efficacy in treating epilepsy compared to the classic keto diet (D'Andrea Meira et al., 2019; García-Rodríguez & Giménez-Cassina, 2021; Guzmán & Blázquez, 2004).

This paper describes the development, optimization, characterization, and preliminary testing of an in vitro model of epilepsy that can be used in nutritional studies and for the testing of keto diets aiming to reduce the adverse effects of epilepsy. The model is created using cells derived from primary rat E18 cortices (Surplus material provided by Utrecht University) containing all naturally present cell types in the cortex at this time point of foetal rat development. We aimed to create an accurate cell culture model that mimics behaviour of cells during states of epilepsy, models network activity in the cortex of the brain, and consists of a similar cell type makeup compared to the in vivo cortex as described in literature.

The cell culture model consists of cells seeded from dissociated cortex material, that typically stays in culture for 15-16 DIV to allow for proper network formation. The short culture duration allows for high throughput assays and frequent testing of both properties of the culture (e.g., medium type, medium changes, coating, the use of different epilepsy models) as well as beneficial effects of nutritional supplements.

Due to the primary nature of the source material for the model, cells in the culture are assumed to maintain ratios like in vivo ratios of cells in the cortex. Specifically, neurons, astrocytes, oligodendrocytes, and microglia were quantified, and percentages of these cells are compared to results in literature to determine how close the behaviour of the cells in culture can be related to in vivo conditions of the developing cortex.

In vitro culture models of the cortex have been found to be able to adapt to a ketogenic diet and for energy production of the cells be in large part reliant on ketone body formation in glial cells as opposed to energy production related to glucose delivered by circulation (Silva et al., 2022). This change in metabolism of glucose as primary energy source to using ketone bodies as primary source of energy has been described to take some time under conditions of either starvation of glucose/carbohydrates or depravation of glucose/carbohydrates and replacement with a “keto-like” diet, containing high amounts of fatty acids (Silva et al., 2022). To determine the ability of the developed model to swap from a traditional glucose diet to a keto-like diet this report describes preliminary efforts to change the medium of cultured cells to low glucose variants and media containing ketone bodies to determine its ability to induce ketosis in vitro.

In vitro cultures of the cortex are described to develop strong interconnecting networks characterized by network bursting activity by 10-14 days in vitro (DIV) which can keep maturing until 21 DIV (Weir et al., 2023). That network activity has been documented to portray a form of epileptiform-like activity, which can be modified using a variety of antiepileptic drugs (AEDs) (Boutin et al., 2022; Pacico & Mingorance-Le Meur, 2014) and whose network activity has been found to depend on the inclusion of a variety of different kinds of neurons normally found in the cortex, including interneurons and projection neurons (Boutin et al., 2022; Pacico & Mingorance-Le Meur, 2014).

Two assays were used to develop, optimize, and characterize the culture method. Immunofluorescence (Staining) is used to characterize and quantify the contents of the cultures. Neurons, astrocytes, oligodendrocytes, and microglia were quantified using fluorescently labelled antibodies and the results were compared to literature. Additionally, characterization of the culture happened by staining with markers for GFAP (Glial Fibrillary Acidic Protein, a marker for reactive Astrocytes) to determine astrocyte morphology and activation state, Iba-1 (a marker for reactive microglia), Parvalbumine (a marker for GABAergic interneurons), and NMDA (a marker for Glutamatergic projection neurons). The results of the quantification and characterization of the staining were used to explain the results of the second assay.

The calcium-6 assay, developed by Molecular Devices, allows for easy and accurate real-time measuring of network activity of the neuronal networks in culture. The principle behind the assay is to replace the medium of the cultures with a calcium-6 dye dissolved in a recording buffer (HBSS +/+) and to record the emitted fluorescent light of internalized dye molecules as the network internalizes the calcium the dye is bound to. This can be measured in real time and if network bursts occur a big peak in emitted fluorescent light can be registered using a FLEX station system in real time. Using the FLIPR machine we record a variety of data about the calcium oscillations of the cultures in question. This data includes peak count/frequency, peak amplitude (an average measure of calcium included in network bursts, giving an indication of the size of the network and/or its ability to pull calcium into the cells), and area under the curve (total intracellular calcium or total activity across a certain timespan) among many other characteristics of the network activity of the cells. The calcium assay can also be used to study the effects of antiepileptic drugs on the different cells participating in the network bursts. This has previously been studied in literature and is included here as a control to determine if the neurons in our cultures are susceptible to having their calcium signalling altered under epileptic conditions (Boutin et al., 2022; Pacico & Mingorance-Le Meur, 2014).

This study consists of three distinct parts. The first is the development of the model and the optimization of culture conditions. We looked at the culture media (compositions, combinations of seeding and growth media), medium changes during the culture period (to determine if the cells can handle medium changes without affecting timely network formation, a requirement to study a swap in diet from carbohydrate-rich to lipid-rich (Calderón et al., 2017; Guzmán & Blázquez, 2004; Ji et al., 2020; Silva et al., 2022), plate coating (specifically determining optimal coating between commonly used methods like a combination of Poly-D-Lysine in hydrobromide and sodium borate (Kim et al., 2011) or a similar coating with addition of natural mouse laminin (Zhang et al., 2020), and optimal cell seeding density, which has been found strongly correlated to the cultures ability to create a strongly bursting network in a timely fashion (Pacico & Mingorance-Le Meur, 2014). The second part of the study consists of the creation and validation of a model of epilepsy. Literature describes methods of 4-AP addition (an epileptic behaviour inducing drug (Boutin et al., 2022; Pacico & Mingorance-Le Meur, 2014) and use of a low magnesium recording buffer (Low extracellular magnesium during the assay prevents competition of said magnesium with calcium for the activation of the receptor that pulls calcium into the cell) to induce epileptiform activity in vitro (Pacico & Mingorance-Le Meur, 2014). This report describes attempts at using these models to induce epileptic behaviour in the cortical cultures. The third and final part of the study describes experiments in which nutritional supplementations are administered to the cultures with mixes of nutrients known to promote growth of cells in culture and alternative sources of energy (low glucose media, ketone bodies) to establish if the assays are sensitive enough to pick up such changes in culturing conditions.

Methods and materials

Source material

Surplus E18 rat cortices from four different animals were collected every Monday morning from the biology department at the Utrecht University. The cortices were stored and transported to Danone Nutricia Research facilities in chilled hibernate-E (Gibco) supplemented with 1% Penicillin/Streptomycin (P/S, Gibco, 10,000 U/mL Penicillin, 10,000 ug/mL Streptomycin).

Plate coating

Plates were coated the Friday before seeding. 96-well culture plates (Perkin Elmer Viewplate 96 F TC, tissue culture treated, clear bottom, black, polystyrene) were coated with a mixture of 10 ug/mL Poly-D-Lysine hydrobromide (PDL, Sigma-Aldrich, P6407), and 50 mM (1x) of borate buffer (20x Pierce borate buffer stock, Thermo Fisher Scientific, 10638294) in sterile demineralized H2O (dH2O), for 1 hour at room temperature. The plates were then washed with dH2O 4 times to remove any traces of borate buffer. The plates were dried under airflow inside the laminar flow cabinet until completely dry and then stored at room temperature over the weekend. Alternatively, plates with a Natural Mouse Laminin coating (Laminin/LAM, Gibco, Laminin mouse protein, Thermo Fisher Scientific, 10267092) were incubated over the weekend with a mixture of 2 ug/mL Laminin in DMEM Low Glucose (Gibco, +1 g/L D-Glucose, + Pyruvate) at 4-8 ^{o}C.

Seeding

On DIV 0 the plates were placed in the incubator to heat to 37 ^{o}C. Hemisphere cortices from all four animals were pooled, washed, and dissociated using a mixture of enzymes and buffers from the Neural Tissue Dissociation Kit (Miltenyi Biotec) in a heated gentleMACS octo dissociator (Miltenyi Biotec). After the dissociation procedure, the cells were filtered through a 40-um cell strainer (Fisherbrand) and afterwards washed with 10 mL of HBSS +/+ (Gibco). The cells were spun down for 5 minutes at 1100 rpm and resuspended in 2 mL fresh warm Neurobasal medium (NB, Gibco, - L-glutamine) supplemented with 2% B27 (Gibco, 50x stock), 1% P/S, and 1% GlutaMAX (Gibco, 100x stock). The cells were counted using a fully automated Vi-cell XR counter (Beckman Coulter, cell viability analyser). Cells were then diluted and seeded in appropriate density (20,000 or 50,000 cells per well) in fresh warm seeding media, either in NB medium or NeuroCult (NC, NeuroCult neuronal plating medium, Stem Cell Technologies) medium supplemented with 1% P/S, 2% NeuroCult SM1 supplement (SM1, NeuroCult SM1 neuronal supplement, Stem Cell Technologies), and 1% GlutaMAX. The outermost ring of wells within the plates was filled with 200 uL of PBS to prevent evaporation-related issues.

Cell culture overview

Plates seeded with cells were incubated at 37^{o}C at 5% CO2 for the entirety of the culture period. The culture period for experiments was until DIV 15 or 16 depending on the need for supplementary interventions on DIV 14. On DIV 3, all cells receive 100 uL of growth medium. Growth media used included NB medium, Myelination medium (MYM, a custom-formulation myelination-inducing medium based on DMEM low glucose from Lariosa et al. 2018 ((Lariosa-Willingham & Leonoudakis, 2018; Lariosa-Willingham et al., 2016), see supplementary), or BrainPhys (BP, Stem Cell Technologies) supplemented with 2% SM1 and 1% P/S. Cells were then left until DIV 15 or 16 when plates were either fixed with PFA for immunofluorescence, or incubated with calcium-6 dye buffer in preparation of a calcium-6 assay. Cells that were kept in culture for DIV 16 received one more medium change (100 uL of medium removed, 100 uL fresh medium added) with fresh growth media on DIV 14 to study the impact of a late medium change on network formation and network activity.

Calcium-6 assay

A recording buffer (dye buffer) for the calcium-6 assay is prepared by dissolving a vial of Component A calcium-6 dye powder from the Calcium-6 assay kit (Molecular devices, kit includes component A calcium-6 dye powder, and component B recording buffer) in 10 mL of Calcium-6 assay Component B buffer and diluting with 10 mL of HBSS +/+ with 20 mM HEPES (Gibco, 100x stock) added. The medium on the cells is replaced with warm recording buffer. Cells were incubated with the recording buffer for 2 hours to allow for good uptake of the calcium dye in the cells. The FlexStation 3 machine (Molecular Devices, FLIPR system) is preheated until 37^{o}C. A typical run was either 334 or 667 reads long over 5 to 10 mins per column (6 wells per column measured at any time). The interval between measurements was set to 0.9 seconds. Further settings included: FLEX mode, fluorescent light emitted at 525 nm, excitation at 485 nm and a single cut-off at 515 nm wavelength. PMT at medium. Flashes per read set to 1. For experiments where compounds were added during the run, a v-bottom source plate needs to be prepared and clean tips placed in the FlexStation 3. For these experiments, a single addition of twenty-two uL was set to occur at the halfway point of the run. Results of the calcium-6 assay were analysed in the SoftMax Pro 7.0 software (Molecular devices). Compounds evaluated included 4-Aminopyridine (4-AP, potentiates voltage-gated Ca^{2+} channel currents, 33 uM), Bicuculline (GABA-antagonist, 10 uM), CNQX (AMPA antagonist, 25 uM), and MK801 (NMDA antagonist, 25 uM).

Fixing and Immunofluorescence

Plates were fixed with a solution of 8% PFA (Thermo Fisher Scientific) in medium (1:1) for 15 minutes. The PFA mixture is discarded, and the cells were washed with PBS 4 times. Plates are stored at 4-8 ^{o}C. A 2x blocking buffer consisting of 78% antibody buffer (150 mM NaCl, 50 mM Tris base, 1% BSA, and 100 mM L-Lysine in dH2O), 20% Normal goat serum, and 2% TX-100 (10% stock) is used to block the culture for 1 hour at room temperature and as a solvent for primary and secondary antibodies. Cells were incubated with primary antibodies dissolved in blocking buffer overnight at 4-8 ^{o}C. The cells were washed with PBS 3-5 times to remove any traces of primary antibodies on the cells. The cells were then incubated with secondary antibodies dissolved in 2x blocking buffer at room temperature for 1 hour. The cells are washed again and a 1:10000 solution of Hoechst (20 mM stock, 33342) in PBS is placed on the cells. The plates are stored at 4-8 ^{o}C with an airtight anti-evaporation plate seal until imaging.

Primary antibodies used include antibodies against NeuN (Chicken host, Millipore, 1:1000, ABN91, Polyclonal), Olig2 (Mouse host, Merck, 1:500, AB9610, Polyclonal), Sox9 (Rabbit host, Millipore, 1:500, AB5535, Polyclonal), GFAP (Rabbit host, DAKO cytomatic, 1:1000, Z0334, Polyclonal), Iba-1 (Rabbit host, WAKO, 1:1000, 019-9741, Polyclonal), NMDA (mouse host, Abcam, 1:100, ab2533060, polyclonal), Parvalbumine (rabbit host, Abcam, 1:200, ab11427, polyclonal), and MBP (Rat host, Abcam, 1:4000, ab7349, polyclonal). Secondary antibodies used are mentioned in supplementary.

Microscopy

Imaging was done using a ImageXpress micro confocal microscope (Molecular devices) and MetaXpress software (Molecular devices). Images were taken with a 10x widefield lens. Nine images at different sites were taken per well, at four different wavelengths. Hoechst/DAPI, 488 FITC, 594 Texas Red, and 647 Cy5 wavelengths were all imaged for every site. Analysis was set up and performed using the Custom Module Editor (CME, Molecular Devices) included in the MetaXpress software. A custom module was created to quantify nuclei count for Sox9, NeuN, and Olig2, and a separate module was created to quantify Iba-1-positive cell bodies.

Results

Method development and optimization – Cell type quantification

To quantify total cells, neurons, astrocytes, and oligodendrocytes in culture at the endpoint of the experiments an analysis method was created to qualify and quantify cells after immunofluorescent staining. Cells labelled with Hoechst (total nuclei count), NeuN (neurons), Olig2 (oligodendrocytes), and Sox9 (expressed by, among other cell types, astrocytes) were imaged and counted using the ImageXpress and MetaXpress software. Using its built-in Custom Module Editor an analysis method was created to quantify these cell types based on marker expression, staining intensity and cell/nucleus size. Figure 7 shows the results of immunofluorescent staining of a primary E18 neuronal culture for core cell types of the central nervous system, as well as an overview of the results of the analysis method and an overlay of all different cell types. All images in Fig. 1 show the same culture, with Hoechst recorded on the DAPI wavelength, NeuN recorded on the FITC (488) wavelength, Sox9 recorded on the Texas Red (594) wavelength, Olig2 recorded on the Cy5 (647) wavelength.

Fig. 1A shows a culture labelled with Hoechst to mark all cell nuclei. Fig. 1B shows a culture labelled with NeuN to mark post-mitotic neurons. Fig. 1C shows the result of analysis with the custom module editor to quantify the different cell types in culture. Fig. 1D shows a culture labelled with Sox9 to mark astrocytes. Fig. 1E shows a culture labelled with Olig2 to mark Oligodendrocytes. Fig. 1F shows an overlay of Hoechst, NeuN, Sox9, and Olig2 stainings.

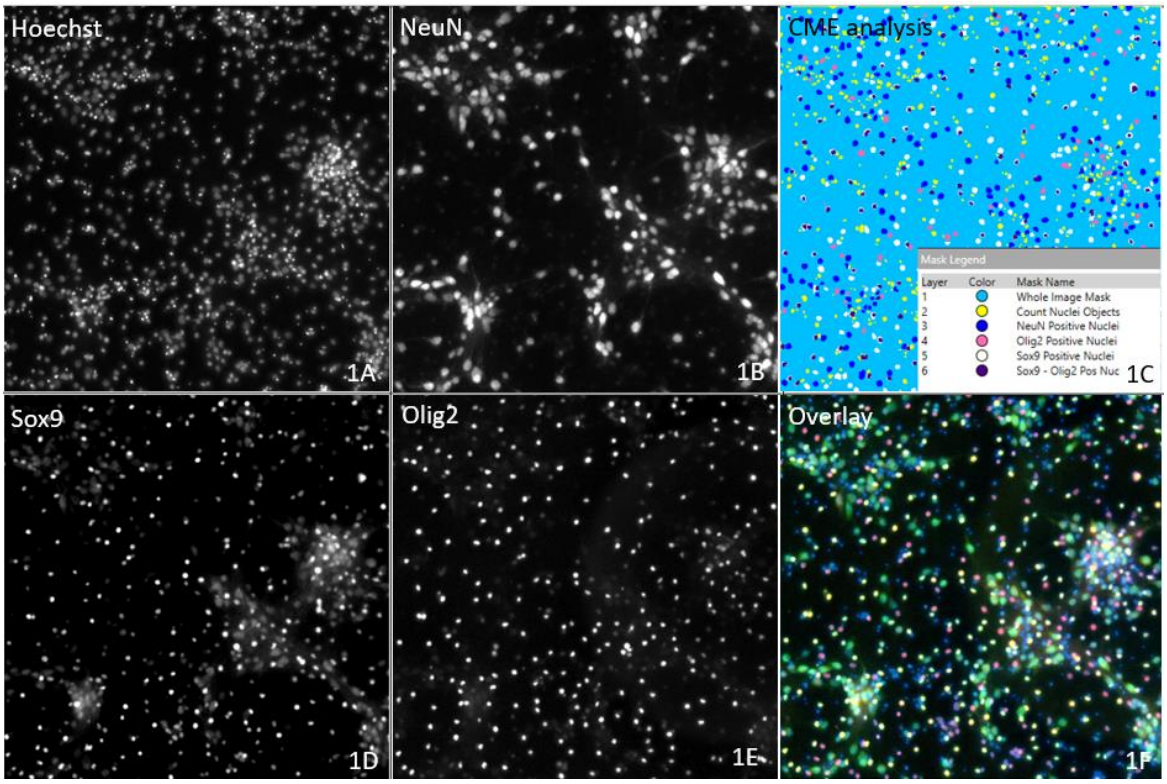

Fig. 1; Overview of images representative of stainings for primary cell types of the central nervous system, and analysis method to quantify these cell types. Includes neurons, astrocytes, and oligodendrocytes as well as total nuclei/cell count 1A. Hoechst dye staining to label all nuclei, recorded at DAPI wavelength. 1B. NeuN immunofluorescent staining to label post-mitotic neurons, recorded at FITC 488 wavelength. 1C. Quantitative analysis of all labelled cell types using custom module editor in MetaXpress. 1D. Sox9 immunofluorescent staining to label astrocytes and oligodendrocytes, recorded at Texas red 594 wavelength. 1E. Olig2 immunofluorescent staining to label oligodendrocytes, recorded at Cy5 647 wavelength. 1F. Overlay of Hoechst (blue), NeuN (green), Sox9 (red), and Olig2 (yellow) stainings. All images reflect the same part of an image taken at 10x magnification.

Method development and optimization – Cell seeding density and medium change protocol

To compare the impact of cell seeding density and the frequency of medium supplementation on network development and resulting network activity we performed an experiment where cells were seeded in NB medium in densities of 20,000 and 50,000 cells per well, and with two different medium change schedules. The plates for this experiment were coated with Poly-D-Lysine + Hydrobromide (PDL) + natural mouse Laminin (LAM) coatings. This combination of coatings is called LAM in the graphics. All cells were seeded in 100 uL NB medium. On DIV 3 the cells were supplemented with another one hundred uL of medium (NB or MYM). The two protocols then differ from that point on. Based on the protocol of (Pacico & Mingorance-Le Meur, 2014), half of the medium on the cells was replaced every other 3-4 days. This protocol was called OSC. For the other protocol, called MYE and based on the work of (Lariosa-Willingham & Leonoudakis, 2018), nothing changed to the medium after the DIV 3 supplementation until the calcium oscillations were measured on DIV 15. Figures 2A and 2B show the results of this experiment, reflecting both the count for network bursts, described as Peak Count, and the average amplitude of the bursts measured reflecting the ability of the cells in the network to pull calcium into the cells during an oscillation, as measured with the calcium-6 assay. Peak count and Peak amplitude were the most common outcome measures during calcium-6 assays for this study. Figure 2C shows a typical recording of the calcium-6 assay for a single well, to give an indication of how peak count and amplitude are recorded or described.

Fig. 2A shows that the count of the network bursts increases when cell count is increased from 20,000 to 50,000 cells seeded for all culturing conditions. This is in line with findings from (Pacico & Mingorance-Le Meur, 2014) which also suggest that increased cell seeding density enables the formation of a stronger network with a higher bursting frequency. Since neurons do not typically divide, a critical number of neurons might be required for the formation of a strong network, although it could also be related to a lack of cellular proximity or release of enough growth factors to create an environment capable of developing a strong network. The Myelination medium change protocol (MYE) also shows increased peak count for cultures with 50,000 cells compared to cultures grown using the oscillation (OSC) medium change protocol. Fig. 2B shows similar differences between cultures seeded with 20,000 and 50,000 cells. The average peak amplitude was much higher for cultures grown in NB medium seeded with 50,000 cells. The results for cultures grown in NB MYM were inverse, with a decrease in average peak amplitude when comparing cultures seeded with 20,000 cells compared to 50,000 cells. Cultures grown using the MYE medium change protocol showed a decrease in average peak amplitude for cultures seeded with 50,000 cells compared to cultures grown using the OSC medium change protocol. This is a common finding when comparing high frequency bursting networks; the average peak amplitude goes down when the peak frequency increases. A possible explanation for this is that the frequency becomes so high that not enough extracellular calcium can be pulled into the cell for every oscillation in a rate-limiting fashion. Fig. 2C depicts a typical measurement of a single well in the calcium-6 assay. Peak count refers to the number of peaks that cross a predetermined amplitude threshold. The average amplitude refers to the intensity of peaks in RFU, calculated as the RFU value at the tip of the peak minus the RFU value at baseline of the peak.

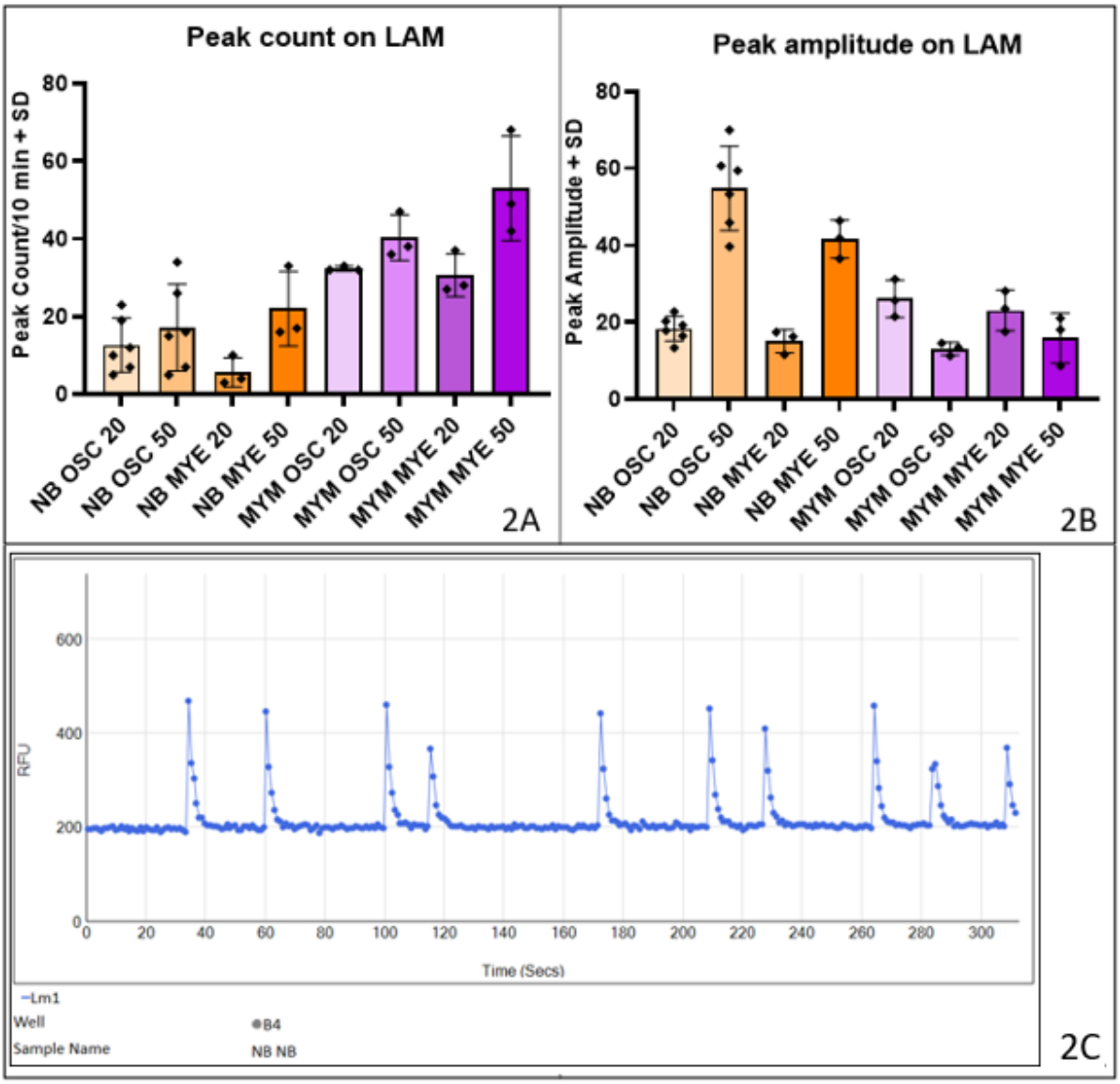

Fig. 2; A comparison of cell seeding density and medium change protocol for cultures. Cell seeding density (20,000 and 50,000 cells seeded) and medium change protocol (OSC = every 3-4 days, MYE = no medium changes) were compared on LAM coating with cells in NB and MYM media conditions. N = 3-6 wells per condition. 2A. Peak count measurements on PDL-LAM-coated wells, comparing media type, cell seeding density and medium change protocol. 2B. Peak amplitude measurements on PDL-LAM-coated wells, comparing media type, cell seeding density and medium change protocol. 2C. Typical readout of a single well for the calcium-6 assay. Error bars indicate standard deviation.

The data from the experiment above indicates that cultures with 50,000 cells seeded show stronger network activity and burst count compared to cultures seeded with 20,000 cells. Additionally, the average peak amplitude shows increases for cultures grown in NB medium for both medium change protocols between 20,000 and 50,000 cells and decreases for cultures grown in the MYM medium. Based on the above we conclude that cultures with 50,000 cells create networks with more synchronized activity, and that the myelination medium change protocol leads to higher peak frequency compared to cultures grown with the oscillation medium change protocol. Based on these findings the following experiments will all be seeded with 50,000 cells and cultured using the myelination medium change protocol.

Method development and optimization – Coating

To determine the optimal cell culture plate coating an experiment was set up to compare two of the most used coatings for the culture of primary neuronal cultures. A coating with Poly-D-Lysine-Hydrobromide (PDL) in a borate buffer was compared to a similar coating with an additional incubation with Natural Mouse Laminin (LAM). Cells were seeded in a NB seeding medium and then supplemented with growth media (NB, MYM, or BP media) on DIV 3. The cells were then used in calcium-6 assays to determine coating impact on network maturation and activity, and were stained using antibodies against NeuN, Olig2, and Sox9 to quantify differences in neuron, oligodendrocyte, and astrocyte count as well as total cell count based on coating differences. Figure 3A and 3B show peak count and peak amplitude respectively for NB, MYM, and BP media on both PDL and LAM coatings. Figure 4 A-F show results of the staining of these cultures with Hoechst (total nuclei count), NeuN (mature post-mitotic neurons), Sox9 (a marker we use to quantify Astrocytes), and Olig2 (Oligodendrocyte lineage marker). Astrocytes were quantified by counting Sox9-positive cells and counting Sox9+ plus Olig2+ cells (Double positives), and withdrawing double positives from the Sox9+ cells, as the double positive cells are considered oligodendrocytes and not astrocytes.

The data (Fig. 3A + 3B) in this experiment indicate that Peak count and Peak amplitude are intrinsically linked as properties. The peak counts of cultures grown in NB medium are similar on both coatings, and so are amplitudes. NB BP shows decreased peak count from LAM to PDL, and increased amplitude, respectively. In the NB MYM media, the opposite conclusion can also be confirmed, as higher peak count leads to lowered amplitude. Fig. 3A shows LAM and PDL coatings do not have consistent effects on bursting frequency/count. The coatings do seem to have effects on the peak frequency within a medium condition, but this was not consistent between experiments (data not included). Fig. 3B shows peak amplitude for PDL and LAM-coated wells under varying media conditions. No clear conclusions can be drawn from peak amplitude between coatings used.

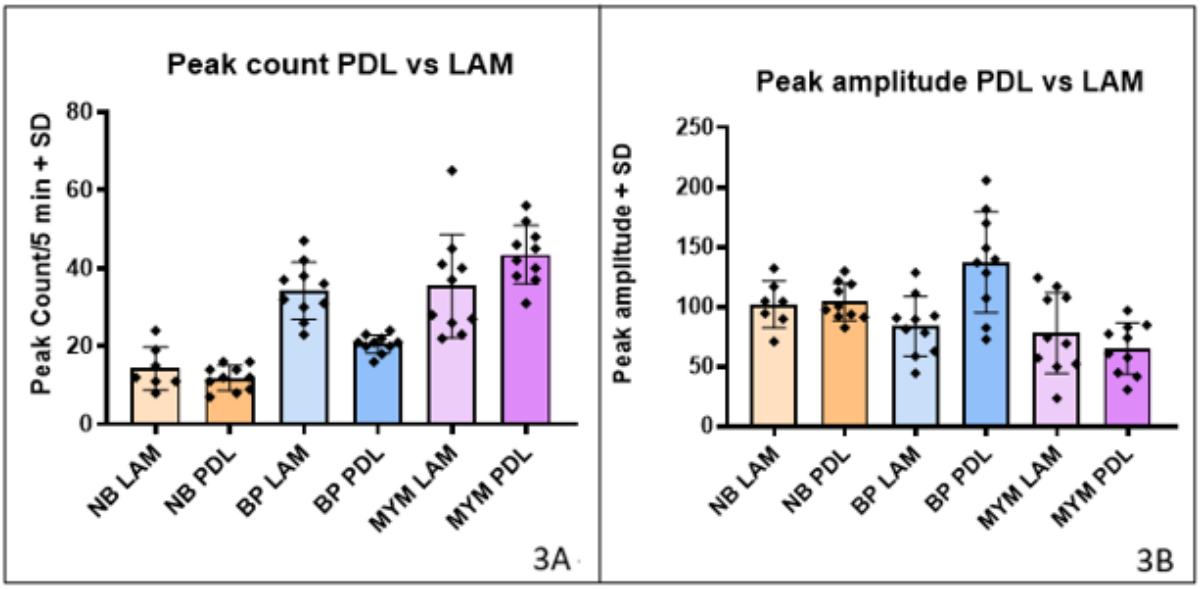

Fig. 3; A comparison of LAM and PDL coatings on three different media as resulting from a calcium-6 assay. N = 10 wells per condition. 3A. Peak count comparison of PDL and LAM-coated wells with NB, NB MYM, and NB BP media. 3B. Peak amplitude comparison of PDL and LAM-coated wells with NB, NB MYM, and NB BP media.

Fig. 4A shows total nuclei quantified by Hoechst dye staining. PDL shows increased cell growth compared to LAM-coated cultures. Neurobasal (NB) cultures have the highest total cell count and NB BrainPhys (NB BP) cultures have the lowest cell count. This can potentially be explained due to high D-glucose content in the NB medium. Fig. 4B shows percentages of total stained cells compared to total nuclei (100%). The results are consistent, which means that cell growth/survival between media types and coatings are constant and do not increase the numbers of stained cells more so than the unstained cells remaining. Percentages of stained cells are stable around 60% although other experiments show numbers as high as 75% stained (data not shown). Fig. 4C-E show total numbers of cells labelled for NeuN, Olig2, and Sox9 respectively. NeuN numbers vary between media types (likely due to different levels of neuronal survival) but are consistent between coatings. This is explainable by the fact that NeuN marks post-mitotic neurons that cannot divide. Olig2 positive cells are strongly increased on PDL compared to LAM-coating for NB and Myelination (MYM) media, but not as strongly for BP. This could be explained due to BP medium being designed to support optimal growth of neurons, possibly resulting in reduced proliferation of other cell types. Fig. 4F shows positive count for perceived astrocytes which are decreased in LAM compared to PDL and much lower for NB BP compared to the other media.

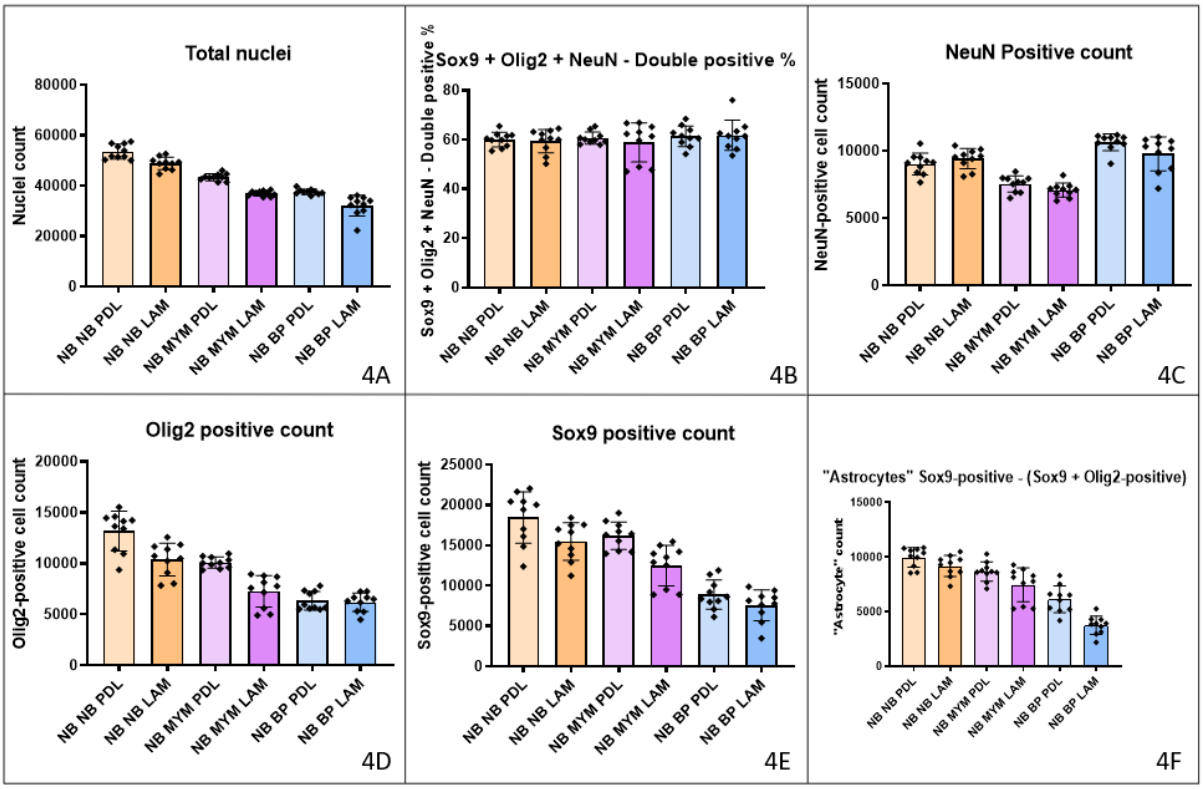

Fig. 4; A comparison of PDL-, and LAM-coatings on various media as compared via Immunofluorescent staining using antibodies against NeuN (Neurons), Olig2 (Oligodendrocytes), Sox9 (Astrocytes) and Hoechst dye (total nuclei count). N = 10 wells per condition. 4A. Total nuclei as quantified by analysing the results of dye staining with Hoechst. 4B. The sum of all stained cells (Sox9 + NeuN + Olig2) set out as a percentage compared to total nuclei counted (the result of 3A). Double positive cells with Olig2+ and Sox9+ are not counted twice. 4C. NeuN positive cell count. 4D. Olig2 positive cell count. 4E. Sox9 positive cell count. 4F. "Astrocytes" as quantified by subtracting Sox9+/Olig2+ cells from all counted Sox9+ cells.

The data from the experiments above indicate increased growth on PDL-coated wells, which could alter the ratio of cells present in the cultures as compared to data reported in vivo. Based on these findings we made the decision to chose to continue culturing only on LAM coated plates.

Method development and optimization – Medium

An experiment was set up to determine the optimal medium to be used for the development of the epilepsy model. Four different medium compositions, Neurobasal (NB), Neurobasal Myelination (NB MYM), Neurobasal BrainPhys (NB BP), and NeuroCult BrainPhys (NC BP), are compared in these experiments. The differences in neuronal network activity of the cultures are compared using the calcium-6 assay to give an indication of the ability to form a stable functional network in culture. Additionally, staining is performed to characterize the cultures and determine how culturing conditions affect the ratios between major cell types as well as how these ratios compare to literature of in vivo material. The cells were dyed with Hoechst and stained for NeuN, Olig2, and Sox9. Figures 5A-D show an overview of typical oscillatory patterns across cultures grown using different medium compositions as recorded during the calcium-6 assay. Figures 6A and 6B show results for peak count and average peak amplitude of the calcium-6 assay for all four media compositions. Figures 7A-F shows the results of staining for NeuN, Olig2, Sox9 and Hoechst for all media combinations. To make a clear comparison between the media, the focus of the graphs in figure 7 is on percentages of the different cell types compared to total nuclei.

Fig. 5A shows a typical oscillatory pattern and recording of the calcium oscillations of a culture grown in NB medium. It is characterized by few peaks, with an inconsistent peak spacing and varying peak amplitude. Fig. 5B shows a typical oscillatory pattern and recording of the calcium oscillations of a culture grown in NB MYM medium. It is characterized by high frequency and low amplitude peaks. Peaks tend to overlap and have varying amplitude. Fig. 5C shows a typical oscillatory pattern and recording of calcium oscillations of a culture grown in NB BP medium. It is characterized by stable frequency with consistent peak spacing and peak amplitude. Fig. 5D shows a typical oscillatory pattern and recording of calcium oscillations of a culture grown in NC BP medium. It is characterized by stable frequency with consistent peak spacing and amplitude, like NB BP cultures. Its peaks are typically sharper with a shorter peak width. This indicates a quicker depolarisation cycle of the neurons in the network and a faster response of the cells in the network.

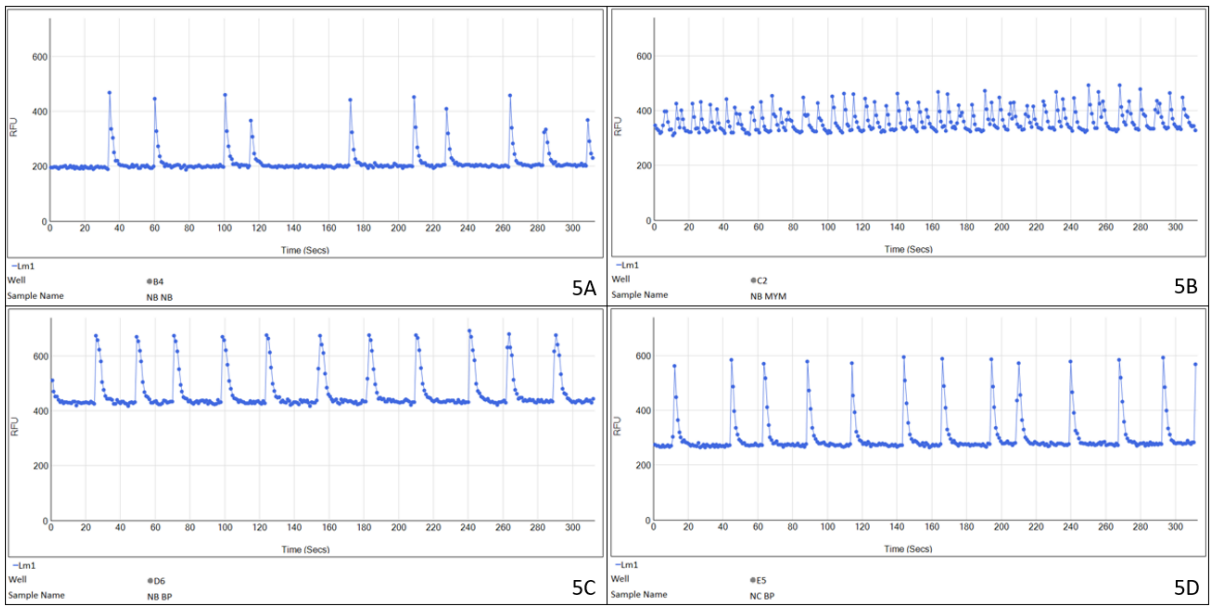

Fig. 5; An overview of typical oscillatory patterns as recorded for the different cultures in a calcium-6 assay. Comparison of NB, NB MYM, NB BP, and NC BP medium conditions. 5A. Oscillatory pattern of a NB culture. 5B. Oscillatory pattern of a NB MYM culture. 5C. Oscillatory pattern of a NB BP culture. 5D. Oscillatory pattern of a NC BP culture.

Fig. 6A shows the peak count of cultures with all four media compositions in the calcium-6 assay. Variance is quite high within every condition, with the least amount of variance seen within the NC BP condition, which has very consistent peak frequency across the cultures. Peak frequency is lower for cells cultured in NB, compared to cells cultured in NB MYM, NB BP, and NC BP. The peak count data for MYM is at times incorrect due to a combination of peak amplitude being so low that not all peaks get recorded, and peak count being so high that peaks overlap. This results in total peak count being higher than recorded in the results of Fig. 6A. Fig. 6B shows average peak amplitude for all cultures. The average amplitude shows high variance within cultures. NB MYM cultures have low average peak amplitude compared to the other culturing media. The BrainPhys media have the highest amplitude and high frequency to create cultures with robust network activity compared to cultures grown in NB and NB MYM media.

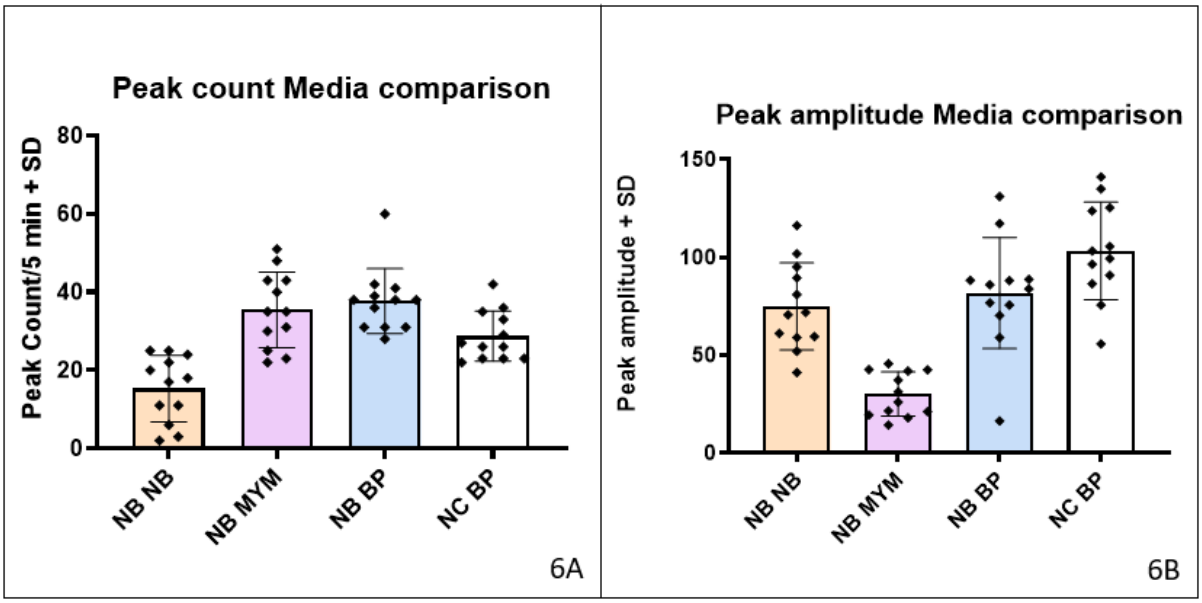

Fig. 6; A comparison of all media type combinations on performance in the calcium-6 assay. N = 10 wells per condition. Comparison of NB, NB MYM, NB BP, and NC BP medium conditions. 6A. Peak count across different media compositions. Error bars indicate SD. 6B. Peak amplitude across different media compositions. Error bars indicate SD.

Fig. 7A shows total nuclei stained by Hoechst. NB cultures show a clear increase in cell count compared to cultures grown using the other medium compositions. Fig. 7B shows percentages of cells stained compared to total nuclei (100%). Like in previous experiments, this number is somewhere around 60% for most cultures. This indicates that differences in growth between cultures affect all cells in culture, not just the core cell types of the central nervous system. Fig. 7C shows NeuN cell percentages compared to total nuclei. BP cultures have the highest percentages of neurons. NB cultures have the lowest percentage of neurons. Total neuron numbers are stable between cultures, with MYM cultures showing slightly lower numbers of neurons compared to cultures grown in different media (data not displayed). Fig. 7D shows Olig2 cell percentages compared to total nuclei. NB cultures have the highest percentage of Olig2+ cells, and NB MYM cultures the lowest percentage of Olig2+ cells. Fig. 7E shows comparable trends for Sox9 cell percentages compared to Fig. 7D. NB cultures have the highest percentages of sox9 positive cells, and cultures grown in other media compositions have 5-10% lower numbers. Fig. 7F shows a strong increase in Astrocytes in line with the increase of Sox9 positive cells for cultures grown in NB, with cultures grown in the other media conditions again not showing big differences between each other.

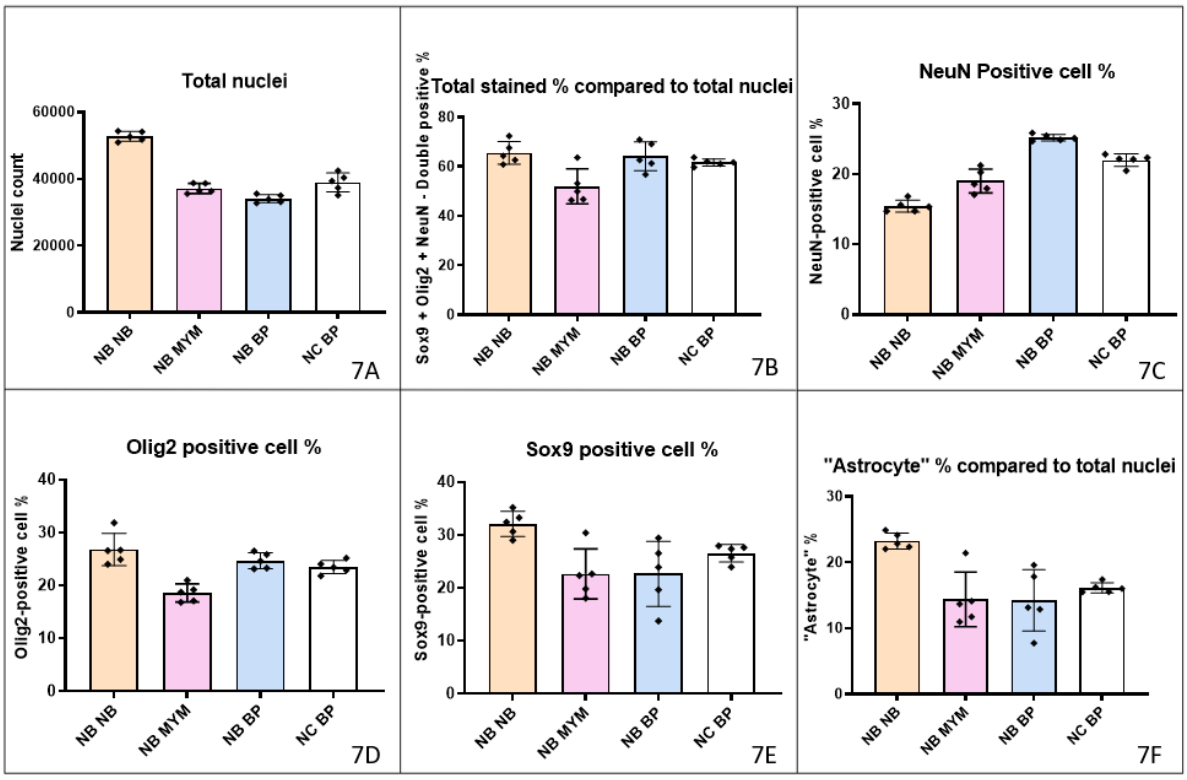

Fig. 7; A comparison of all media type combinations on culture characterization using immunofluorescent staining. N = 5 wells per condition. Comparison of NB, NB MYM, NB BP, and NC BP medium conditions. 7A. Total nuclei as quantified by analysis of Hoechst dye staining. 7B. Percentage of all cells stained across every medium condition. Sox9 + NeuN + Olig2 – Double positive Sox9/Olig2 cells compared to total nuclei. 7C. Percentage of cells positive for NeuN. 7D. Percentage of cells positive for Olig2. 7E. Percentage of cells positive for Sox9. 7F. Percentage of "Astrocytes" as defined as Sox9+ - (Sox9+ + Olig2+) cells. Percentages from graphs 5C-F are compared to total nuclei. All error bars indicate standard deviation.

The calcium-6 assay shows that the networks of the BrainPhys media are the most robust, with consistent and high peak frequency, and high amplitude. For the staining, NB cultures show increased cell count compared to the other media conditions. NB cultures show increased percentages of Olig2, and Sox9-positive cells compared to cultures grown in the other media compositions. The BrainPhys cultures have high percentages of neurons and stable populations of Sox9 and Olig2 positive cells.

Method development and optimization – Culture characterization

To determine the presence of other cell types beyond neurons, oligodendrocytes, and astrocytes, an experiment was set up to confirm the presence of microglia. For this, Iba-1 was used to label the microglia. Additionally, to characterize the neurons, astrocytes, and oligodendrocytes in culture, stainings were performed for NMDA (glutamate receptor marker), Parvalbumin (subtype of GABA receptor marker), MBP (Myelin-basic protein, protein product of oligodendrocytes used in myelin sheath formation), and GFAP (to show morphology of the Astrocytes). Figures 8A-F show the results of staining with these markers.

Fig. 8A confirms presence of Microglia in culture at DIV 1. The culture was fixed and stained at DIV 1 because microglia were not found at DIV 15+. It is quite common for microglia to either lose their markers because of an altered environment ex vivo (suggesting the cells remain alive in culture, and can possibly be stained with different markers), or to die due to lack of relevant growth factors like M-CSF1, IL34, and TGF-B (Bohlen et al., 2019; Bohlen et al., 2017). The cell count for microglia was low, at 0.3 – 0.6%. Literature suggest that it could be as high as 5% in vivo (Dos Santos et al., 2020; Keller et al., 2018). Fig. 8B shows staining with an antibody against Parvalbumin. Fig. 8C shows staining with an antibody against NMDA. Fig. 8D shows staining for GFAP in a NB MYM culture. There is a lot of GFAP expression, and the morphology is strongly ramified. Fig. 8E shows staining for GFAP in a NB culture, and the expression is much lower compared to the NB MYM culture but keeps the same ramified morphology. Fig. 8F shows staining for MBP in a NB MYM culture of 20,000 cells. It shows strong streaking of axons and protein deposits. Cultures with 50,000 cells show no MBP formation.

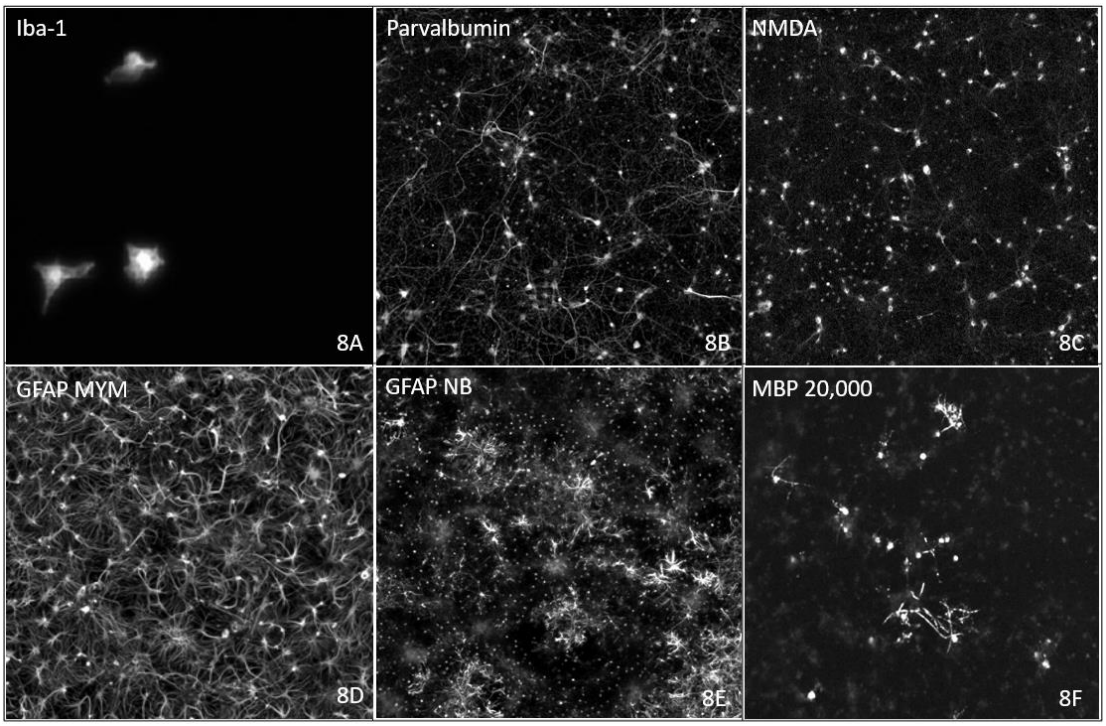

Fig. 8; Summary of efforts to characterize the culture with markers against microglia (Iba-1), GABA receptor expressing neurons (Parvalbumin), Glutamate receptors (NMDA), Astrocytes in cultures of NB and MYM (GFAP), and myelin basic protein (MBP) formation. 8A. Microglia on DIV 1 (20x). 8B. Neurons labelled with Parvalbumin, a marker for a subset of GABA receptor expressing neurons commonly found in the cortex (10x). 8C. Neurons labelled with NMDA, a marker for a specific glutamate receptor (10x). 8D. a MYM culture stained with GFAP to visualize activated astrocytes (10x). 8E. a NB culture stained with GFAP to visualize activated astrocytes (10x). 8F. Myelin basic protein deposits and axon-wrapping (20x). The cells in all figures except for the microglia in figure 8A are fixed on DIV 15.

Method development and optimization – Medium changes

To create an accurate model for epilepsy we need to evaluate whether the cultures we are comparing can have their medium refreshed without disturbing development and formation of a strong electro physiologically active network. The cultures need to be able to have their medium changed to a version of a keto diet to study the response of the cells to the convulsant conditions of an epilepsy model. However, it is also well documented that cells require glucose to properly settle during seeding. To determine if the different cultures described in figures 4 and 5 are capable of handling such a medium change, an experiment was set up to compare cultures without intervention after the DIV 3 medium supplementation to cultures with a 50% medium change on DIV 7. Additionally, to determine if medium of cells can be swapped to low glucose variants (to study low carbohydrate conditions) an experiment was set up to study NB culture medium changed on DIV 10 and 14 to low glucose. Figures 9 and 10 show the results of a calcium-6 assay and staining respectively for the comparison of cultures with and without a DIV 7 medium change. Figure 11 shows the results of a calcium-6 assay and attempts at changing neurobasal medium on DIV 10 and 14 to fresh high or low glucose compared to controls. Figure 12 shows the results of a calcium-6 assay and NC BP cultures' ability to resist medium changes with different volumes at different time points. For all medium changes, the medium was replaced with warm fresh growth medium (i.e., the medium that was supplemented at DIV 3, not a combination of both seeding and growth media).

Fig. 9A shows the peak count of different media compositions with and without medium change on DIV 7. A DIV 7 medium change for cultures grown in NB and NC BP media does not affect peak count. Cultures grown using NB MYM and especially NB BP show large reductions in peak count after the medium change. This indicates that the networks of those cultures are less developed and require more time to develop in conditioned medium. Fig. 9B shows average peak amplitude. Cultures grown using NB, NB MYM, and NC BP have consistent peak amplitude or even small increases, however cultures grown in NB BP have almost no activity after the medium change.

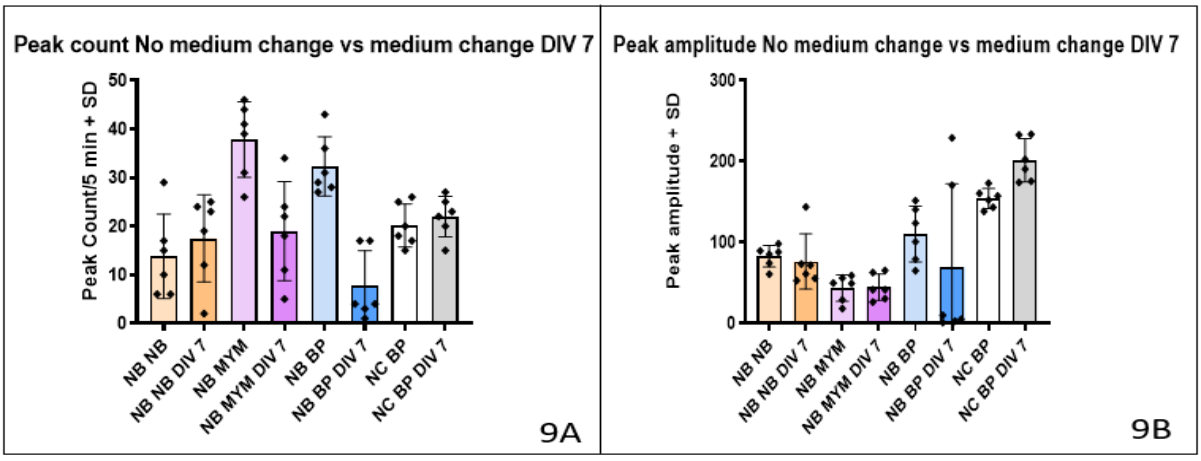

Fig. 9; A comparison of different media compositions with and without a DIV 7 medium change to fresh growth medium. N = 6 wells per condition. 9A. Peak count from calcium-6 assay for different media combinations with and without DIV 7 medium change. 9B. Peak amplitude from calcium-6 assay for different media combinations with and without DIV 7 medium changes. Error bars indicate standard deviation.

Fig. 10A shows total nuclei count as determined by analysis of Hoechst staining for all culture conditions with and without DIV 7 medium changes. The medium change on DIV 7 shows little effect on total cell count for cultures grown in NB MYM, NB BP, and NC BP. Cultures grown in NB BP media show decreased total cell count after a DIV 7 medium change. Fig. 10B shows percentages of cells stained with the markers NeuN, Sox9, and Olig2. This ranges anywhere between 60-80%, with NC BP cultures having the most cells accounted for. Interestingly, most cultures do not show a change between percentages of cells that are stained with and without medium change. Cultures grown in NB do have an increase in stained cells after a medium change on DIV 7. This could indicate that the new NB medium that is added on DIV 7 causes increased growth for oligodendrocytes and astrocytes that are not seen in the other cultures. This is likely because of the high D-glucose levels (25 mM) in this medium compared to the other growth media. Fig. 10C shows NeuN positive cell count and no changes in numbers are seen for any condition. It is not expected to see increases in NeuN count as those cells cannot proliferate. Fig. 10D shows Olig2 positive cell count. Olig2 positive cells are strongly increased in cultures grown using NB and decrease in culture grown in NB BP media. Fig. 10E shows Sox9 count. Sox9 positive cells are also increased for cultures grown in NB and show decreases for cultures grown in NB BP and NC BP. Fig. 10F shows an approximation of total astrocyte count defined as Sox9 positive cells minus cells double positive for Sox9 and Olig2. A minor increase for NB logically follows from increased numbers of Sox9 and Olig2 cells, and a decrease for NC BP can be explained by dropping Sox9 numbers.

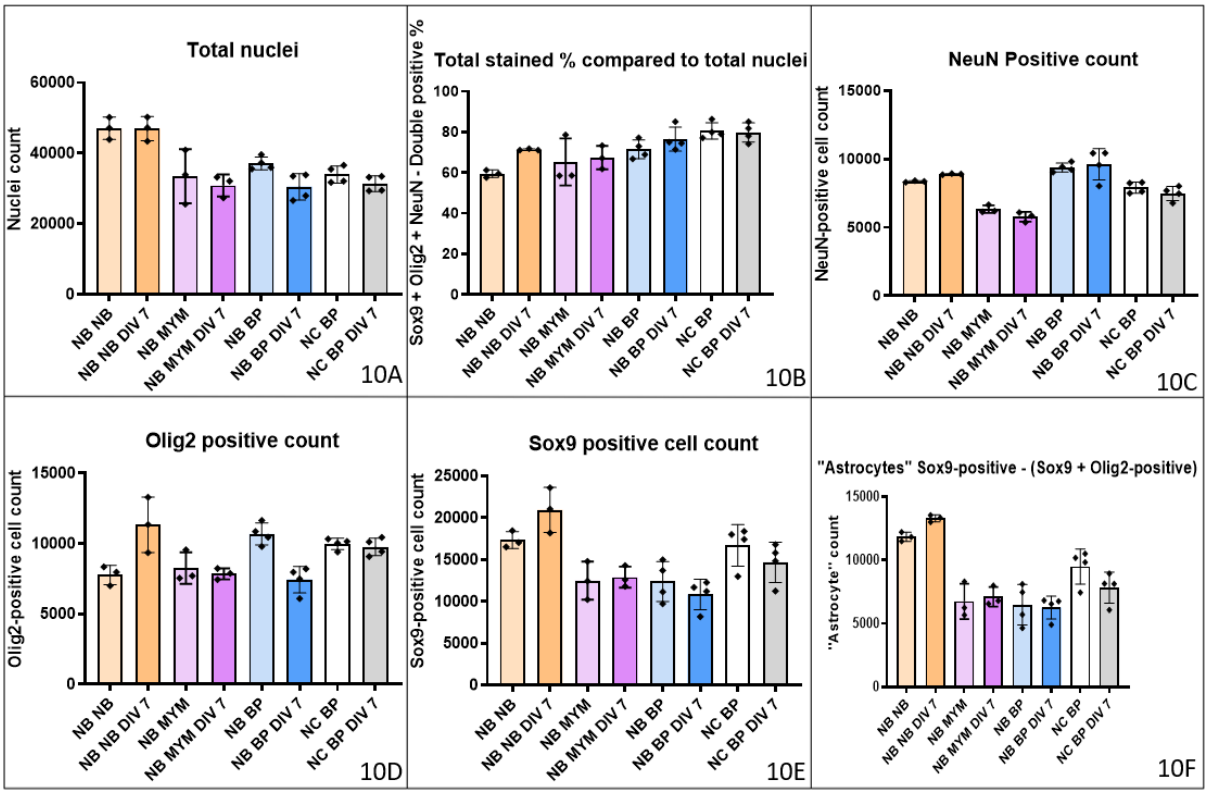

Fig. 10; A comparison of all media type combinations with and without medium change on DIV 7 as resulting from immunofluorescent staining for Sox9, NeuN, and Olig2. N = 3-4 wells per condition. 10A. Total nuclei by medium composition with and without medium change. 10B. Percentages of stained cells compared to total nuclei. 10C. NeuN positive count. 10D. Olig2 positive count. 10E. Sox9 positive count. 10F. "Astrocyte" count as defined by Sox9+ - (Sox9 + Olig2 double positive cells). Error bars indicate standard deviation.

Fig. 11A shows the peak count results of a calcium-6 assay for cultures grown using NB medium with and without a medium change on DIV 10. The medium changes are performed to high and low glucose (LG, see axes of graphics in fig. 11) medium (NB 25 mM D-glucose vs NB-A 0 mM D-glucose). The peak count is not altered on a DIV 10 medium change to medium with either glucose level and no large differences in variance are seen for peak count. Fig. 11B shows the peak count results of a calcium-6 assay in NB cultures with and without medium change on DIV 14. Again, medium changes are described for high and low glucose levels. Here the medium changes on DIV 14 clearly interfere with the ability of the cultures to oscillate and a drop in peak count is seen for cultures undergoing a medium change to high and low glucose medium. Fig. 11C shows average peak amplitude for the cultures with a DIV 10 medium change. No consistent changes in peak amplitude are seen. Fig. 11D shows average peak amplitude for the DIV 14 cultures. Paired with the drop in peak count numbers seen in fig. 11B are big decreases in peak amplitude. Especially the cultures receiving a medium change to low glucose medium show a strong decrease in average peak amplitude.

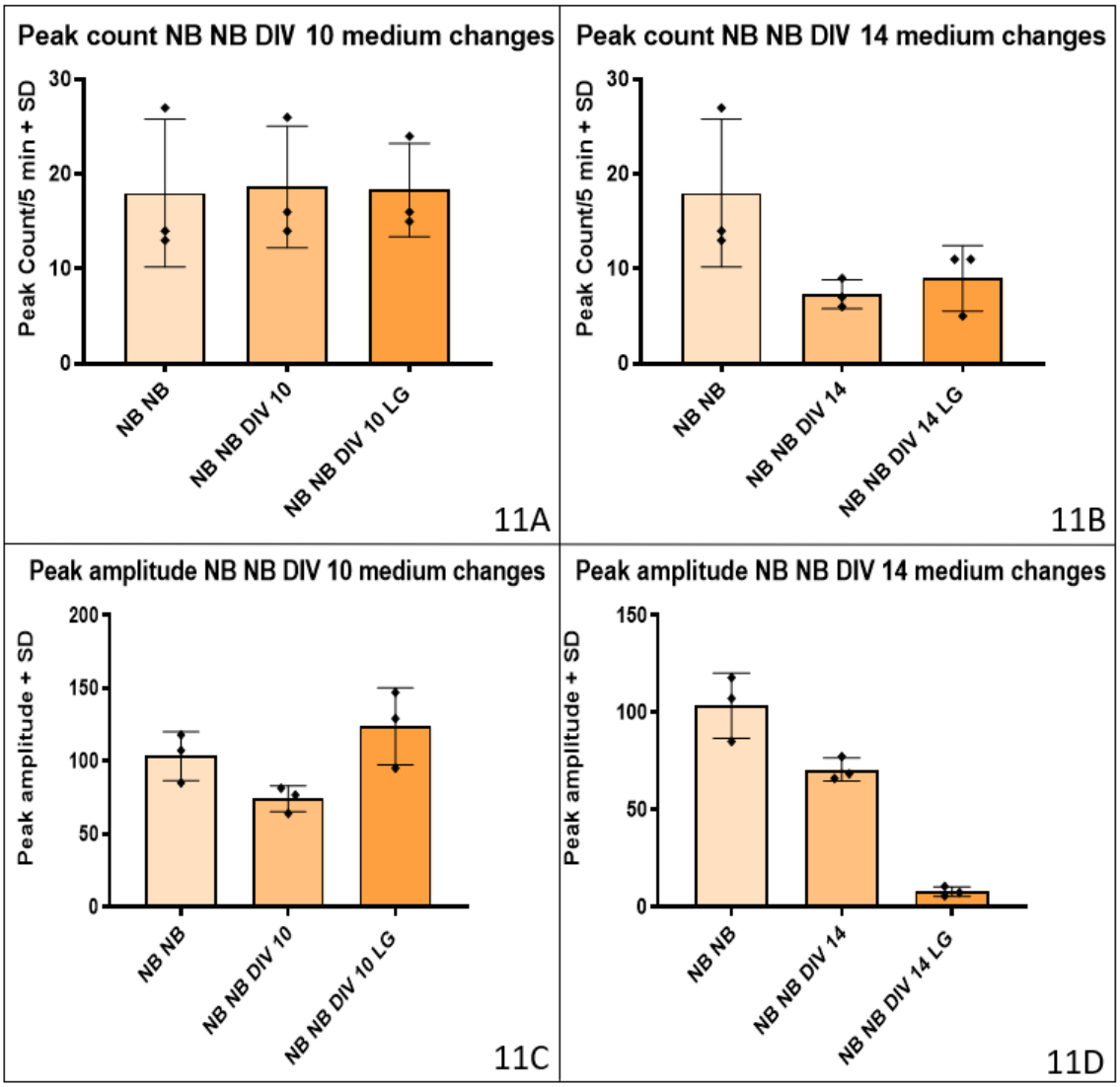

Fig. 11; A comparison of medium changes in NB cultures across DIV 10 and 14 to high and low glucose medium as reported from a calcium-6 assay. N = 3 wells per condition. 11A. Peak count for NB cultures with medium changes to high and low glucose medium on DIV 10. 11B. Peak count for NB cultures with medium changes to high and low glucose medium on DIV 14. 11C. Peak amplitude for NB cultures with medium changes to high and low glucose medium on DIV 10. 11D. Peak amplitude for NB cultures with medium changes to high and low glucose medium on DIV 14. Error bars indicate standard deviation.

Fig. 12 A shows peak count for cultures grown in NC BP with medium changes at DIV 7, 10, and 14. The volume of the medium changes also varied between 50, 75, and 100%. Cultures that received a medium change at DIV 7 (50% refreshed) show inconsistent peak count. Increasing the amount of medium changed to 75% or 100% at DIV 7 results in strong decrease in oscillations. The 50% medium change on DIV 10 has much more consistent frequency compared to the DIV 7 medium change. Despite this, the 75% and 100% conditions still do not show many oscillations. On DIV 14 however, the 50% and 75% both show high peak count with low variance, and the 100% condition also shows activity, although much lower compared to the other conditions. This experiment indicates that the cultures need conditioned medium to mature the network and that conditioned medium is still required for normal network behaviour and activity after network maturation has fully completed. Fig. 12 B shows average peak amplitude for cultures grown in NC BP with medium changes. Cultures with a high peak frequency also show high amplitude for most conditions, except for the cultures that received a 50% medium change on DIV 7.

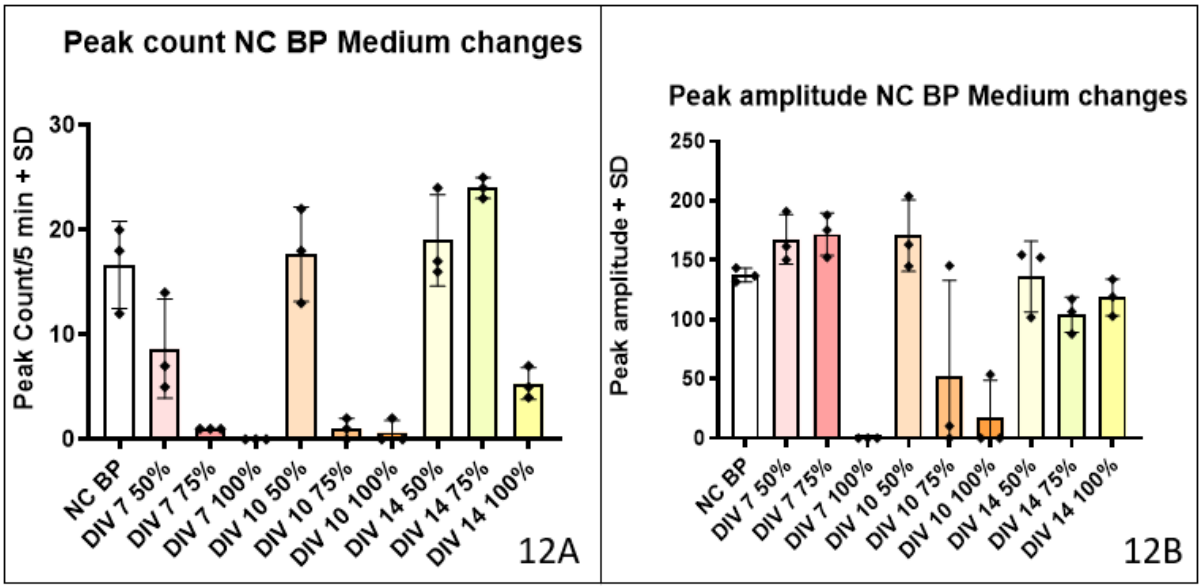

Fig. 12; A comparison of NC BP cultures with medium changes across DIV 7, 10 and 14 in volumes of 50, 75, and 100%. N = 3 wells per condition. 12A. Peak count for NC BP cultures with a variety of medium changes at different timepoints and with differing volumes. 12B. Peak amplitude for NC BP cultures with a variety of medium changes at different timepoints and with differing volumes.

The data from the experiments described above (Fig. 9, 11, 12) suggests that medium changes affect the ability of networks to oscillate. Neuronal cultures seem to need conditioned medium during the early parts of culturing to create a network capable of synchronized oscillations. A late medium change (DIV 14) also seems to hurt the ability of culture to oscillate. This is likely because the cultures are given too little time to recondition the medium before the calcium oscillations are measured on DIV 16. The optimal window for a medium change is likely somewhere in between DIV 7 and 14.

Method development and optimization – Culture duration

To study the role culture duration has in the development of a strong network and to determine if cultures can last this long on a single medium supplementation at DIV 3, an experiment was set up to study the best performing medium compositions in long term cultures. Specifically, rather than DIV 15/16, the cells would be used for a calcium-6 assay at DIV 18 and DIV 21. The cultures for this experiment include NB, NB MYM, NB BP, NC BP, and NC BP medium with medium changes at DIV 7 or 14. Figure 13 shows the peak count and amplitude results of calcium-6 assays for a variety of cultures on DIV 18 and 21 timepoints.

Fig. 13A shows peak count for cultures at DIV 18. All cultures behaved like how they would on DIV 15 or 16. Cultures grown in NB had inconsistent peak frequency, and cultures grown in NB MYM had high frequency. All cultures grown using different BP media conditions had very consistent peak count. Fig. 13B shows peak count for cultures at DIV 21. All cultures show good activity at DIV 21. Cultures grown in NB had high frequency compared to those measured at earlier timepoints. The cultures grown in the other medium compositions similarly all showed an increase in peak count compared to earlier timepoints, albeit not as large an increase. Fig. 13C shows peak amplitude for the DIV 18 cultures. The results here match typical results of a DIV 15 or 16 culture. BrainPhys cultures have the highest amplitude, and MYM cultures the lowest. Fig. 13D shows the peak amplitude for cultures at DIV 21. The cultures grown in BP media still have high peak amplitude, and cultures grown in NB and NB MYM medium have low amplitude.

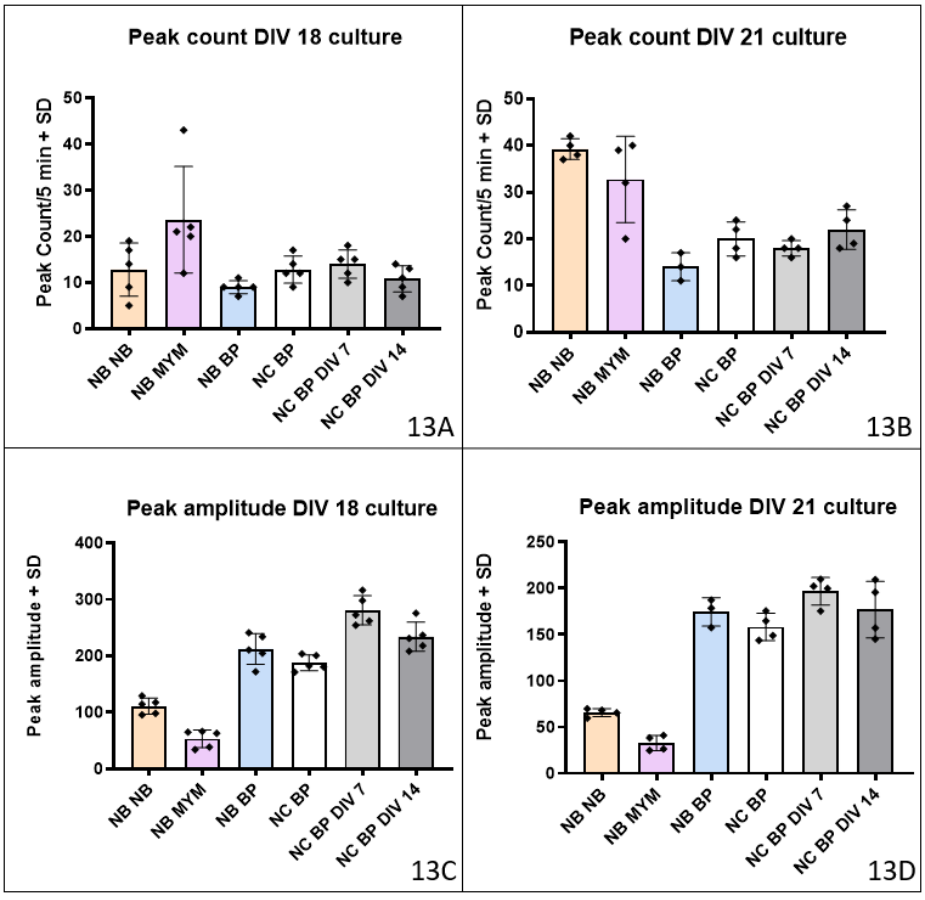

Fig. 13; A comparison of different medium compositions and performance in the calcium-6 assay after long term culture till DIV 18 or 21. N = 5 wells per condition for 11A and 11C. N = 4 wells per condition for 13B and 13D. 13A. Peak count at DIV 18. 13B. Peak count at DIV 21. 13. Average peak amplitude at DIV 18. 13D. Average peak amplitude at DIV 21.

Modelling epilepsy – 4-Aminopyridine

To test whether the cell culture can be used for a modelling epilepsy an experiment was set up to determine the impact of the convulsant drug 4-AP upon addition to cultures with varying medium compositions. 4-AP was added to the cultures during the calcium-6 assay at the halfway point (t=150s) of the run for every well. A stock solution of 330 uM was prepared in HBSS +/+, and 22 uL was injected in every well, for a final concentration of 33 uM 4-AP. Figure 14 shows the area under the curve (total intracellular calcium, or total activity of the culture) of cultures before and after addition of 4-AP. This leads to a direct comparison of network behaviour of the cells in the same wells before and after 4-AP administration. Conditions compared include cultures grown in NB, NB MYM, NB BP, NC BP, and NC BP medium with a DIV 7 medium change.

Fig. 14 A shows total activity of the cells before and after 4-AP addition. The addition of 4-AP causes an immediate change in network activity for every condition. Peak frequency and amplitude become higher and more synchronized. Total activity or area under the curve is strongly increased for every condition. Fig. 14B shows typical behaviour of cells in culture (NB culture in this case) upon activation with 4-AP.

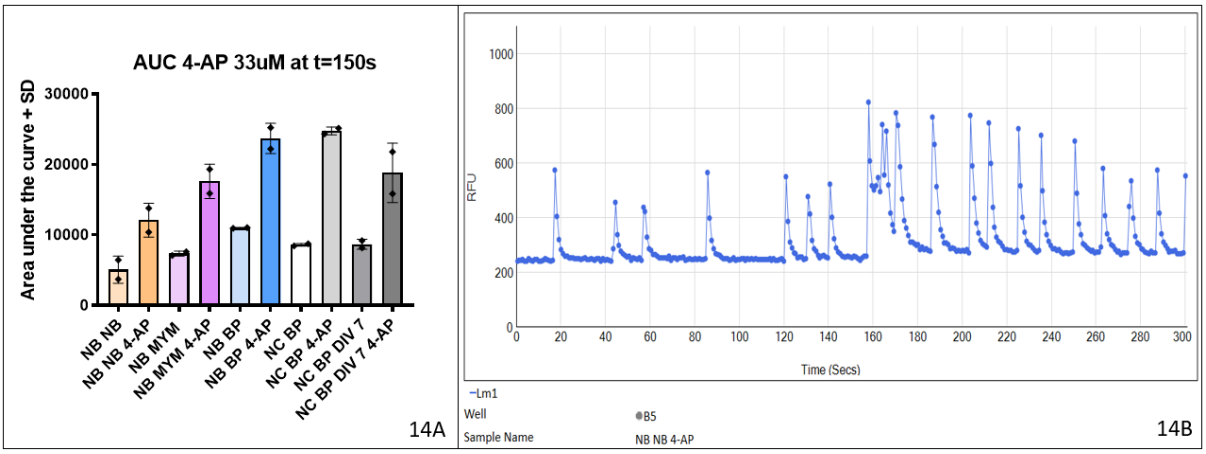

Fig. 14; A direct comparison of wells before and after addition of (a final concentration of) 33 uM of 4-AP at the halfway point (t=150s) of a calcium-6 assay. N = 2 wells per condition. All error bars indicate standard deviation. 14A. Area under the curve (AUC) used as a measure of total activity in the well. 14B. Typical oscillatory pattern of cells activated with 4-AP at t=150s.

Modelling epilepsy – Low magnesium

Another model of epilepsy we wanted to assess is the low magnesium model. This model is employed by replacing the standard recording buffer with a new recording buffer with a 10x dilution of Mg2+ (0.09 mM). The lack of magnesium in the recording buffer prevents competitive inhibition of the NMDA receptor the cells use to pull calcium into the cytoplasm. This receptor can bind Mg2+ thereby competing for activation with Ca2+. Lowered activity of Mg2+ for the NMDA receptor therefore indirectly increases activity of the receptor for calcium and lowers the threshold required for a systemic network burst, increasing peak frequency and stabilizing amplitude. The lowered magnesium recording buffer has a Mg2+ concentration of 0.09 mM as opposed to 0.9 mM in HBSS +/+. Calcium levels are unchanged compared to HBSS +/+ levels. Figure 15 shows the peak count and amplitude of cultures and a comparison to similar cultures with a low magnesium recording buffer.

Fig. 15A shows increases in peak frequency for NB and MYM cultures, but stable frequencies for the BrainPhys media, when the cultures are recorded under low magnesium conditions. Fig. 15B shows average peak amplitude staying stable for the BP and NB media, and a decrease for culture grown in NB MYM medium in line with the increase in peak frequency. Fig. 15C shows the typical oscillatory pattern of a NB culture when incubated with low magnesium recording buffer. It is characterized by increased peak count, and consistent peak amplitude and peak spacing.

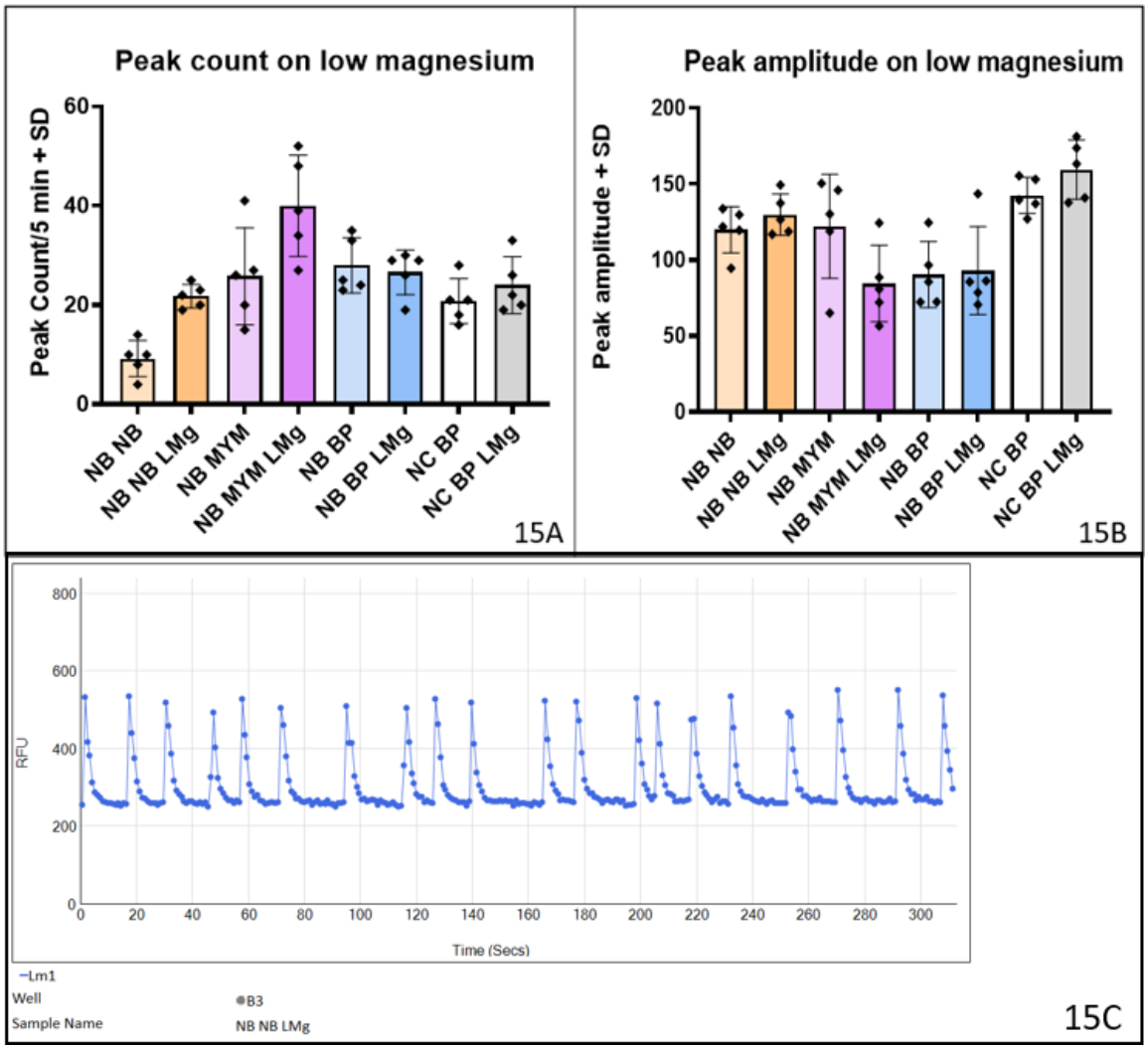

Fig. 15; A comparison for a variety of medium compositions between HBSS +/+ based recording buffer and "Low Magnesium" recording buffer (LMg). N = 5 wells per condition. 15A. Peak count for cultures with and without Low magnesium recording buffer. 15B. Peak amplitude for cultures with and without Low magnesium recording buffer. 15C. Typical oscillatory pattern of cells recorded in low magnesium recording buffer.

The 4-Aminopyridine model of epilepsy shows a strong increase for the total activity of the cells. The oscillatory patterns of all conditions become more erratic, with variances in burst frequency and amplitude. The low magnesium models on the other hand presents with a constant increase in peak frequency and/or peak amplitude. The model only increases activity for cultures grown in NB and NB MYM medium and has no consistent effects on cultures grown in BP medium

Modelling epilepsy – Anti epileptic drugs

To confirm the results of culture characterization and involvement of various kinds of neurons in the neuronal network activity an experiment was set up to test anti-epileptic drugs (AEDs). Drugs assessed were Bicuculline (a GABA receptor antagonist), CNQX (a AMPA glutamate receptor antagonist), and MK801 (a NMDA glutamate receptor antagonist). Like administration of 4-AP previously, the compounds were prepared as a 10x stock and injected with the cells during the calcium-6 assay at the halfway point. This method allows for a direct comparison of culturing conditions in wells with a before-and-after compound addition. Final concentration for Bicuculline was 10 uM in culture, and for CNQX and MK801 was 25 uM in culture. Figure 16 shows the results of a calcium-6 assay with addition of Bicuculline, CNQX and MK801 at the halfway point for a variety of culturing conditions.

Fig. 16A and D show the effects of administration of Bicuculline on peak frequency and amplitude. Fig. 16A shows a decrease in peak frequency for cultures grown in NB and a small decrease for those grown in NB MYM medium, with no effect for cultures grown in the BP media. Fig. 16D shows effects of Bicuculline addition on peak amplitude. The peak amplitude of cultures grown in NB and MYM is raised, and the cultures grown in BP media do not respond like the frequency shown in Fig. 16A. Fig. 16B and E show the effects of administration of CNQX on peak frequency and amplitude. Fig. 16B shows that cultures grown in NB and MYM media have significantly lowered peak frequency, the BP cultures have lowered or unaffected peak counts. Fig. 16E shows effects of CNQX addition upon peak amplitude. Amplitude for all cultures is lowered, although the effects on NB BP seem smaller compared to other cultures. Fig. 16C and F show the effects of administration of MK801 on peak frequency and amplitude. Fig. 16C shows that MK801, like CNQX, lowers peak frequency for cultures grown in NB and MYM media and has varied effects on cultures grown in BP medium. Fig. 16F shows effects of MK801 addition upon peak amplitude. All cultures have significantly lowered amplitude for this condition.

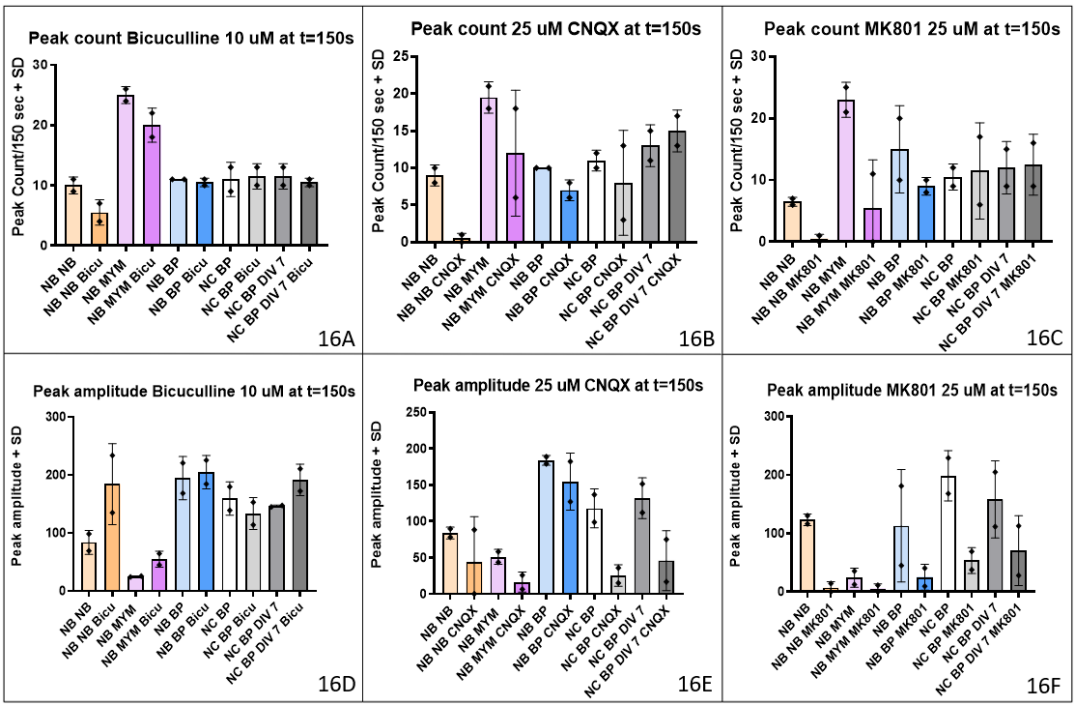

Fig. 16; An overview of the results of a calcium-6 assay performed in various cultures using anti-epileptic drugs. N = 2 conditions per well. 16A. Effect of 10 uM Bicuculline on peak count. 16B. Effect of 25 uM CNQX on peak count. 16C. Effect of 25 uM MK801 on peak count. 16D. Effect of 10 uM Bicuculline on peak amplitude. 16E. Effect of 25 uM CNQX on peak amplitude. 16F. Effect of 25 uM MK801 on peak amplitude. Error bars depict SD.

Supplementation studies – Sodium-3-Beta Hydroxybutyrate (BHB)

To study the effects of BHB supplementation to the cultures and to determine if the calcium-6 assay was sensitive enough to notice such changes in culture conditions an experiment was set up to compare NB cultures with medium changes on DIV 10 and 14 to various media compositions differing in D-glucose levels and BHB supplementation. The final concentration of BHB in cultures was 10 mM.

Fig. 17A shows peak count for NB cultures after medium changes on DIV 10 to high and low glucose medium with and without BHB. A slight trend suggests BHB might increase peak frequency, but the variance is high. It is also clear that the control condition performs poorly, making it unreliable. Fig. 17B shows peak count for cultures receiving a medium change on DIV 14. Here the low glucose media show worse peak count compared to the high glucose medium cultures, and the effects of BHB are not consistent. Fig. 17C shows peak amplitude for the DIV 10 cultures. BHB seems to show a slight positive trend for amplitude. Fig. 17D shows peak amplitude for cultures receiving a DIV 14 medium change. BHB shows a similar trend of increasing peak amplitude, but the amplitude of all cultures receiving a medium change are very weak.

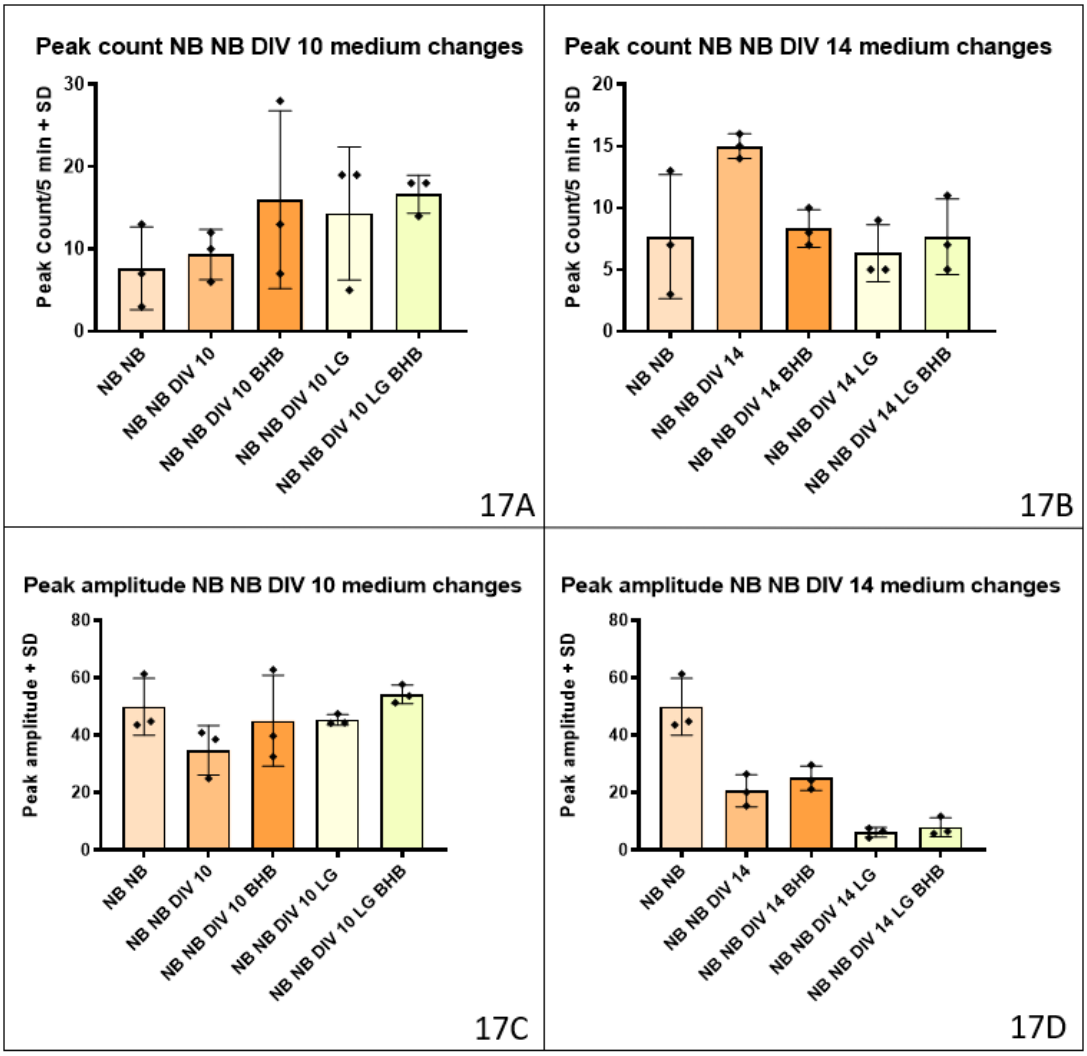

Fig. 17; An overview of NB cultures receiving medium changes on DIV 10 and 14 to Low/High glucose medium with and without 10 mM BHB supplementation. N = 3 wells per condition. 17A. Peak count for NB cultures with medium changes on DIV 10. 17B. Peak count for NB cultures with medium changes on DIV 14. 17C. Peak amplitude for NB cultures with medium changes on DIV 10. 17D. Peak amplitude for NB cultures with medium changes on DIV 14. Error bars indicate standard deviation.

Supplementation studies – Nutritional intervention

To study the ability of the cells in the cultures to be used for studies into nutritional supplementation an experiment was set up to determine if the calcium-6 assay was sensitive enough to pick up the effects of nutritional supplementation. A potent mixture of nutrients known to enhance cell growth and neurite outgrowth in vitro was supplemented to the cultures on DIV 3. To determine if the model can be used to quantify effects of this nutrient mixture, cultures with and without this nutritional intervention are compared. To fully determine the effects of the supplementary intervention, both a calcium-6 assay and staining with NeuN, Sox9, and Olig2 were performed. Figure 18 shows the results of nutritional intervention for cell cultures compared to controls in the calcium-6 assay. Figure 19 show the primary results of the staining with controls being normalized to 100% of response and the experimental group with nutritional intervention set out as a percentage against it.

Fig. 18A shows the effects of nutritional intervention for cultures as compared to non-supplemented controls on peak count. Clear increases in networking bursting are seen in every experimental condition. NB cultures show the weakest effect with the most variance, and the responses of NB MYM and NC BP are the biggest. Fig. 18B shows the effects of nutritional intervention on peak amplitude. In line with the findings from Fig. 18A, the amplitude is decreased for every condition to match increased peak frequency.

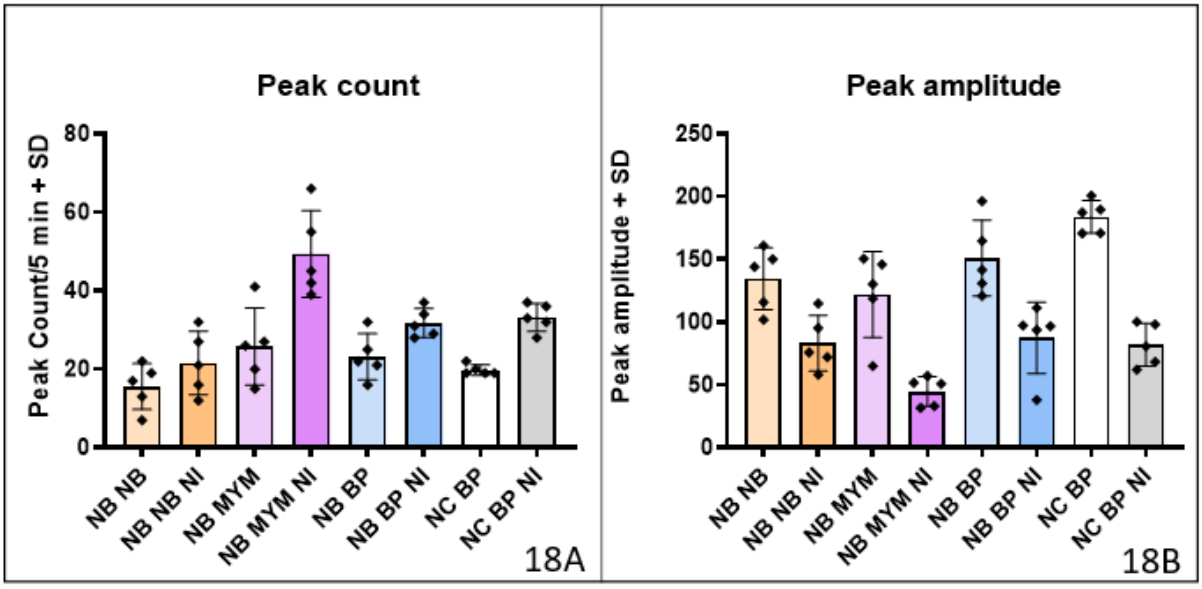

Fig. 18; A comparison between cultures supplemented with nutritional intervention (NI) and controls resulting from the calcium-6 assay. N = 5 wells per condition. 18A. Peak count comparison between cultures supplemented with and without nutritional intervention. 18B. Peak amplitude comparison between cultures supplemented with and without nutritional intervention. Error bars indicate standard deviation.

Fig. 19A shows a clear increase in total nuclei in cultures with a nutritional intervention compared to the controls. Fig. 19B shows constant levels of neurons in culture supplemented with nutritional intervention compared to non-supplemented controls. No clear trends can be seen in the minor differences in neuron numbers, indicating the nutritional intervention does not hurt neuronal survival. Fig. 19C shows strong increases in Olig2+ oligodendrocyte count for cultures grown in all media. Fig. 19D shows that Astrocyte count is stable in cultures grown in NB and MYM media but shows a strong increase in those grown in BP medium. NB MYM cultures have high variance (in comparison to other medium types) in both the control and nutritional intervention experimental groups for all cell specific markers.

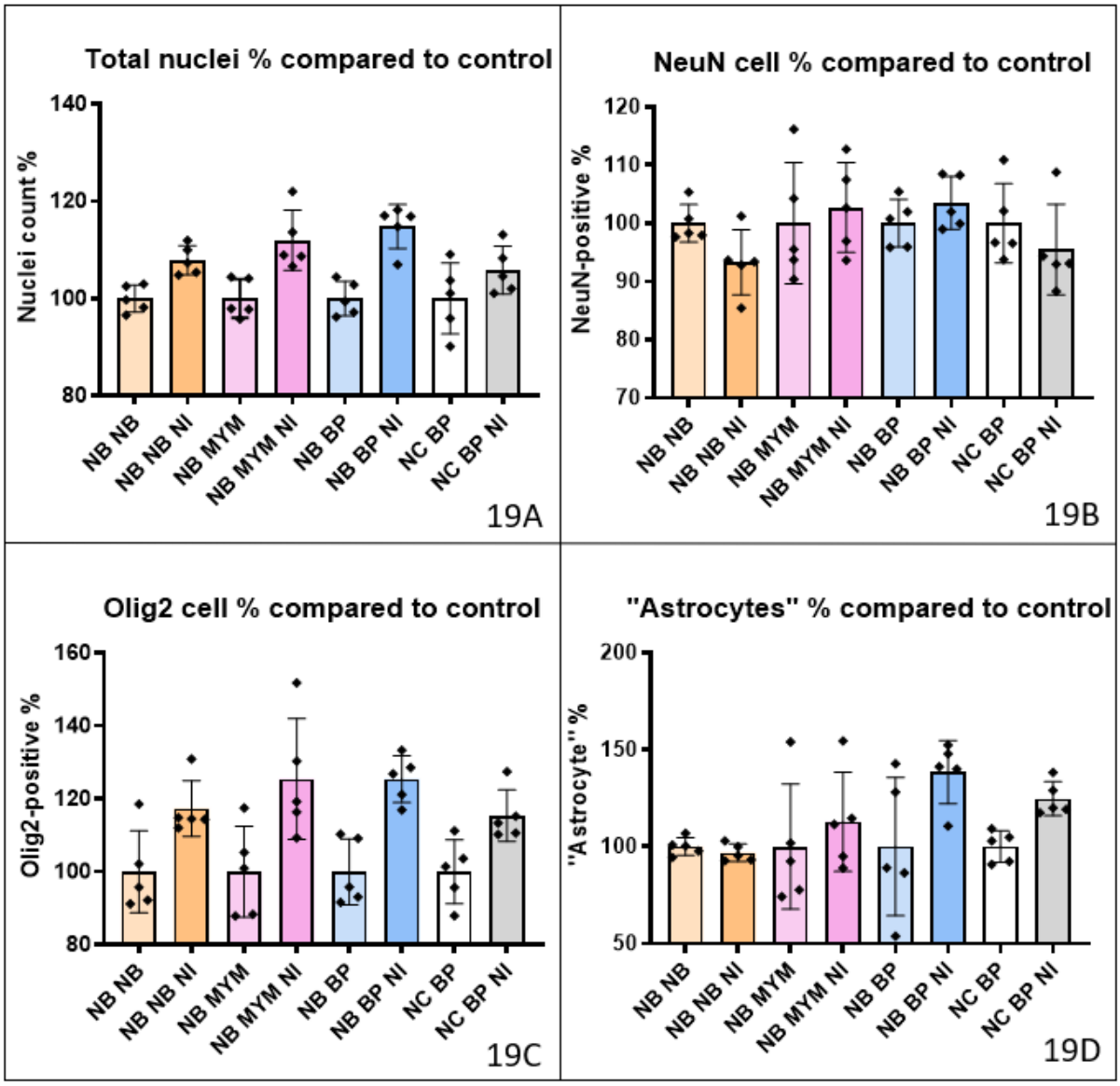

Fig. 19; An overview comparing results from staining on cultures supplemented with a nutritional intervention (NI) compared to non-supplemented control cultures. Average of controls normalized to 100%, experimental nutritional intervention groups set out as percentage to the control. N = 5 wells per condition. 19A. Total nuclei of nutritionally supplemented cultures vs non-supplemented controls. 19B. NeuN positive cell percentages of nutritionally supplemented cultures as compared to normalized control conditions. 19C. Olig2 positive cell percentages of nutritionally supplemented cultures as compared to normalized control conditions. 19D. Astrocyte cell percentages of nutritionally supplemented cultures as compared to normalized control conditions. Error bars indicate standard deviation.

There is a clear window in the calcium-6 assay for every medium composition to study nutritional intervention. It remains to be seen if the effects of individual nutrients can be picked up using the calcium-6 assay. The nutritional intervention has a notable effect on growth of total nuclei and Olig2+ cells in a variety of cultures. Astrocyte count is also increased for BP cultures. These changes in cell count can be used to explain the changes in network activity seen in figure 18.

Discussion

The aim of the study was to develop and evaluate a cell culture-based model for epilepsy that could be used to study modified (via nutritional supplementation) ketogenic diets aimed at preventing and ameliorating disease symptoms. Epilepsy is historically difficult to model in culture because of lacking connectivity seen in traditional neuronal cell cultures (Brofiga et al., 2020). This lack of connectivity can be attributed to alterations in the three-dimensional structure of the cells and the presence of a lowered variety and numbers of cell types in culture compared to in vivo models. Still, in vivo models present with their own complications. Ethical and financial complications make large scale use of animals for high throughput testing of pharmaceutical, surgical, and nutritional interventions difficult. In vivo studies of epilepsy are also complicated due to high variance between individual subjects and models. Culture-based models are by comparison much cheaper and allow for faster high throughput studies to assess for the beneficial effects of pharmaceutical or nutritional intervention (Campos et al., 2018; Raimondo et al., 2017). An accurate in vitro model for epilepsy must cover part of the weaknesses that models based on simple neuronal cultures typically have. These weaknesses include lack of cellular diversity, altered network activity, and poor three-dimensional connectivity. For this reason, we opted to use primary material from the cortex of embryonic rats at E18. The cortex is heavily implied as one of the most important geo-structural locations regarding onset and maintenance of epilepsy in the central nervous system. At E18, the cortex is developed enough to expect to see most important cell types in culture. Primary E18 material comes with the added benefit of having all cell types normally available in the brain in culture, as well as being easy to work with compared to traditional co-culture methods, which lack diversity of cell sub-typing, and require elaborate protocols to keep healthy in culture. Despite this, cellular diversity is not completely alike in vivo cortex material. An example of this is that microglia typically do not survive in cultures unsupplemented with growth factors. Using fresh material every week instead of frozen stocks of purified cells also helps to reduce variability and increase cell viability and normalize gene expression in vitro (Watanabe & Akiyama, 2020). To lower the impact of variability (due to sex, genetic mutations, etc.) of the source material, cortices of four different animals were pooled together in a single cell homogenate.

Two assays were chosen to assess and quantify the ability of this culture protocol to model epilepsy. To measure the network activity of neurons in culture we use the calcium-6 assay developed by Molecular Devices. This assay is simple in execution and design and has minimal impact on the viability or behaviour of the cells in culture. Only a single reagent, the calcium-6 dye, is incubated with the cells. To study neuronal network activity, we measure the occurrence of synchronized network bursting. This phenomenon occurs in cortical and hippocampal primal cultures in vitro when cells in the neural network are allowed to mature for at least 12 to 14 days in culture. At this timepoint the spiking neurons start synchronizing their calcium oscillations resulting in the formation of so-called ‘super bursts,’ where a large part of the network oscillates within the span of 1-3 seconds (Pacico & Mingorance-Le Meur, 2014; Weir et al., 2023). The occurrence of these network bursts relies on good Excitatory-Inhibitory (E/I) network regulation by excitatory glutamatergic projection neurons and inhibitory GABAergic interneurons. As these types of neurons are important for functioning of the cortex and E/I imbalance is heavily implied in epilepsy pathology, these network bursts give a clear and simple to appreciate/interpret overview of the health of the neuronal culture (Ghirga et al., 2021). A large part of this study is assigned to determine which culturing conditions and nutritional supplementations can impact the behaviour of the cells in culture and how they alter the onset of these network bursts. The second assay is characterization and quantification of cell (sub-) types in culture via immunofluorescent staining. These stainings aim to determine the impact of culturing

conditions and nutritional supplementations on cell numbers, types, and variability to use that data to explain the variations we see in the neuronal network activity as assessed by the calcium-6 assay.

To determine what a good model for epilepsy looks like it is important to determine which characteristics and results are considered good, and which are considered bad. For the characterization and quantification of the cell types in culture it would be beneficial good to have a large variety of cell types like in vivo cortex material. Furthermore, having the main cell type numbers and ratios be comparable to the in vivo situation suggests that the cells will respond to nutritional and pharmacological interventions as compared to cells in vivo as well. To this end, quantifying major cell types and characterizing cell subtypes is an important effort towards determining different culturing methods' impact on a translatable model. As mentioned above, these quantifications and characteristics are related to culturing conditions and directly impact the network developing and functioning of the cultures. For the assessment of functional network activity of the cultures it is important to consider which characteristics are associated with good healthy neuronal signalling, and which characteristics are considered potentially pathological or associated with epileptic behaviour. It is well understood that epileptiform behaviour of neurons is characterized by aberrant and uncontrollable electrical signalling between cells, coupled with the lost ability to control this irregular activity via signal inhibition. Conversely, healthy neuronal activity is associated with stable signalling between cells in a well-controlled environment. For this, it is important that excitatory and inhibitory signalling and network activity is well balanced. For the purposes of our calcium-6 assay and based on findings in literature (Ghirga et al., 2021; Pacico & Mingorance-Le Meur, 2014), this translates to stable network burst rates in culture, with steady peak spacing and strong neuron-to-network participation. Additionally, the requirement to use this model for (high throughput-) nutritional studies confirms the need for a stable and consistent network that can detect minor differences in functional behaviour because of minor nutritional interventions.

The first part of this study describes developing the culture model and assessing the impact of differing culture conditions on assay outcome measures and reproducibility.

Cell seeding density has been shown to heavily affect network development, maturation, and network activity in literature (Pacico & Mingorance-Le Meur, 2014) and from our findings in this study as shown in fig. 1. Based on the work of (Pacico & Mingorance-Le Meur, 2014) and (Lariosa-Willingham & Leonoudakis, 2018) we seeded cells at densities of 20,000 and 50,000 cells per well. Cultures of 50,000 cells proved to form stronger networks compared to cultures of 20,000 cells seeded. A logical explanation for this, matching findings in literature (Pacico & Mingorance-Le Meur, 2014), is that higher cell density increases cell-cell interactions and contributes to production of medium conditioned with high levels of growth factors and signalling molecules, which in turn contribute to synaptic maturation. Interestingly, we also found that cultures seeded with 20,000 cells produced more MBP protein deposits (fig. 8F) and even showed preliminary axon streaking, where higher density cultures did not show this protein formation. Something about the lower cell density created for conditions where oligodendrocytes started producing MBP in higher amounts. This is surprising as the seemingly increased maturation of the network should increase oligodendrocyte maturation and MBP formation (Marton et al., 2019). A potential explanation for this is that cultures with 50,000 cells have a different cellular makeup, or that increased access to nutrients (more glucose available per cell) enabled production of more MBP protein product. Despite these findings, cultures with 50,000 cells seemed to mature much more robustly, and since the role of MBP in myelin sheath formation is to enhance signalling, a more robust and active culture network would be preferred over increased MBP formation. As such, we decide to keep culturing with 50,000 cells per well. Increasing cell seeding

count even further could potentially result in the formation of stronger or faster developing networks but also risks negative effects due to competition for resources in the culture medium.

To study the effects of medium supplementation and medium change protocols we compared two protocols based on work of (Pacico & Mingorance-Le Meur, 2014) and (Lariosa-Willingham & Leonoudakis, 2018; Lariosa-Willingham et al., 2016). The protocol based on the work of (Pacico & Mingorance-Le Meur, 2014) refreshes half of the medium of cells in culture every 3-4 days, while the protocol based on the work of (Lariosa-Willingham & Leonoudakis, 2018; Lariosa-Willingham et al., 2016) supplements medium 3 days into the culture and leaves it there until the assays were performed. We compared these culture protocols and found that the single supplementation strategy of Lariosa yielded formation of a much more active and developed culture (fig. 2). Because of these findings we decided to use this single supplementation protocol.

Culture plate coatings have been shown to heavily affect cell behaviour and growth for a variety of cell types. Commonly used plate coatings for neuronal cultures include options like Poly-D-Lysine, Poly-L-Lysine, and natural mouse laminin coatings (Kim et al., 2011; Zhang et al., 2020). Because Poly-L-Lysine coatings are subject to enzymatic digestion (Mazia et al., 1975), we chose to compare a PDL-Hydrobromide in sodium borate buffer coating (PDL) vs a similar coating with an additional incubation with natural mouse laminin (LAM). Across different media compositions we could not find consistent differences for the coatings in the calcium-6 assay. The coatings do not alter network activity and network formation by a large amount. We did find differences in cell type quantification, however. A consistent trend (fig. 3) arose that showed that neuronal cultures on PDL coatings had higher cell count and numbers compared to the LAM coated cultures. Due to consistent numbers of NeuN-positive neurons between the two coatings, which are post-mitotic cells and cannot proliferate, it supports the conclusion that the cells grow (more) on PDL compared to LAM coatings as opposed to the differences being related to survival. Cultures on PDL coated plates show strong increases in the numbers of Olig2+ Oligodendrocytes, Sox9+ astrocytes, and the percentage of stained cells is consistent between PDL and LAM-coated plates. This suggests that the numbers of unstained cells also increase to offset the increase of oligodendrocytes and astrocytes. Additionally, the lower numbers of cells in cultures on LAM coatings could be related to decreased proliferation and increased maturation or differentiation of oligodendrocytes and astrocytes. Based on the finding that PDL promotes growth of specific cell types in the brain in an unpredictable way, we chose to culture on LAM coatings to preserve more accurate ratios of cell types in vivo.

The most impactful culture condition is the medium composition. Based on the previously mentioned protocols of (Lariosa-Willingham & Leonoudakis, 2018; Lariosa-Willingham et al., 2016) and (Pacico & Mingorance-Le Meur, 2014) we culture the cells in respectively neurobasal medium (NB) or in a combination of neurobasal seeding medium with myelination growth medium (MYM) supplemented on DIV 3. BrainPhys medium (BP) based on the work of (Bardy et al., 2015) is also tested as a growth medium as it is supposed to be very potent at increasing network activity and network maturation. For the BP cultures we tried both NB and NeuroCult (NC) seeding medium. The use of different medium compositions has shown large differences in performance of cultures in the calcium-6 assay and in deciding culture cell count and compositions. NB cultures show the biggest cell growth (fig. 6A). NB medium has high glucose content (25 mM) which might be responsible for this trend. The increased glucose levels of NB medium compared to MYM (5.5 mM), and BP medium (2 mM) could explain differences in cell growth or survival. It also shows large increases in numbers of Sox9+ and Olig2+ cells compared to the other media, with around 30% of total cells in this culture for both types of cells (Fig. 7D and 7E, almost 60% accounted). Comparatively, the MYM and BP cultures have significantly lower total cell count (Fig. 6A), with higher percentages of cells being NeuN positive (Fig. 7C, but similar

exact numbers between all cultures, data not included). Especially the BP cultures have high percentages of neurons, as the medium is designed to increase neuronal performance and survival and does not support growth of other cell types due to low glucose content (2mM). The cultures grown in MYM medium have the lowest percentages of olig2+ cells. As the myelination medium increases maturation of oligodendrocytes (Fig. 7D) (Lariosa-Willingham & Leonoudakis, 2018; Lariosa-Willingham et al., 2016) this is likely responsible for the decreased proliferation. For the calcium-6 assay we also found large differences between the medium compositions. NB cultures had low, inconsistent peak frequency, characterized by inconsistent peak spacing, and highly varying peak amplitude (Fig. 6, 5A). MYM cultures had high peak frequency (to the point that peaks would sometimes overlap and be hard to quantify), and low but stable peak amplitude (Fig. 6, 5B). The BrainPhys cultures, seeded on both NB and NC medium instead showed stable mid-to-high frequency peaks, and very consistent peak spacing and amplitude (Fig. 6, 5C+D). Based on these results the BrainPhys media produced the most consistent cultures in line with traits of healthy signalling behaviour.

To determine if the cultures could be used to assess the effects of a ketogenic diet and for nutritional supplementation studies, we also decided to evaluate changing and refreshing the medium during the culture period. Multiple medium changes have shown to impair network formation and maturation as shown in the decision to go for a single medium supplementation on DIV 3 (Fig. 1). Despite those results, the need for a culture that can manage medium changes is important to study a swap from a carbohydrate-rich to a lipid-rich ketogenic diet. Major differences were found between cultures of the different medium compositions in handling medium changes at varying timepoints. NB cultures can handle a DIV 7 or 10 medium change, but network activity is decreased after a late DIV 14 medium change (Fig. 9, 11). Cultures grown in NB MYM as well as NB BP cannot handle a medium change at any point during the culture period (Fig. 9). For those cultures, burst formation was either severely hurt (MYM, low frequency, low inconsistent amplitude) or completely lost (NB BP). NC BP cultures manage medium changes better than the other cultures. A DIV 7 medium change showed a slight reduction in peak frequency but otherwise stable amplitude and good network maturation (Fig. 12). DIV 10 and 14 medium changes showed no differences compared to cultures without medium changes (Fig. 12). Staining also showed interesting alterations in cell numbers between cultures with a medium change at DIV 7 and those without. NB cultures that received fresh new NB medium showed increases in Olig2+ and Sox9+ cells (Fig. 10D, E), likely due to the new glucose added into the culture. NB BP cultures showed a decrease in total cell numbers, as well as Sox9+ and Olig2+ cell count (Fig. 10A, D, E). This decrease in cell count could explain the poor network activity after the medium change. An explanation for this could be that cultures based on NB seeding medium require high amounts of glucose to function properly, and that replacing the NB medium with low glucose media like MYM (~5mM), and BP (2mM) lowers cell viability. Based on these results, NB and NC BP cultures show the highest promise as media compositions for cultures that are used to study ketogenic diets or nutritional supplementation studies.

Literature suggest that these kinds of cultures keep developing their networks for as long as 21 DIV (Weir et al., 2023). To confirm these findings and determine if the cells could stay in culture for longer periods of time on a single medium supplementation on DIV 3, we started an experiment for longer term culture. Plates with cultures on varying media conditions were cultured until DIV 18 and 21 (as opposed to standard DIV 15-16) and evaluated in the calcium-6 assay. On DIV 18, all cultures behaved exactly like they did on DIV 15 (Fig. 13). Interestingly, cultures on DIV 21 still showed good oscillations and behaved exactly like cultures on DIV 15. Interestingly, the NB cultures showed highly increased peak frequency and had much more stable high amplitude (Fig. 13). If this result can be replicated it could indicate that long term culture with NB medium is one of the best possible protocols for cultures

not requiring a late medium change. In addition, cultures show good network activity at DIV 21, indicating the cultures can be used for even longer on the single medium supplementation.

To characterize the contents of the cultures we stained with antibodies against Iba-1 (microglia), GFAP (astrocytes), NMDA (glutamate receptor), and Parvalbumine (GABA receptor). On DIV 15, no Iba-1+ microglia were found in any of the cultures. Upon fixation at DIV 1, Iba-1+ microglia were found in small numbers (Fig. 8A). Quantification showed numbers ranging between 0.3 - 0.6% of total cell count. Literature suggests that microglia numbers should be consistent in the cortex around 5% of total cell count (Bohlen et al., 2017; Dos Santos et al., 2020). These numbers are preserved between species. A few explanations can be found for these findings. Microglia do not typically survive in neuronal cultures without supplementation of IL-34, M-CSF1, and TGF-B (Bohlen et al., 2019; Bohlen et al., 2017; Goshi et al., 2020). Additionally, microglia are prone to lose their cell type specific markers in culture, likely due to altered environment compared to the physiological environment (Goshi et al., 2020; Liu et al., 2021; Yeh & Ikezu, 2019). Knowing that microglia are not in culture but are present in the source material opens opportunities to try and prevent the loss of microglia in the cultures. Growth factor and cytokine supplementation could prevent this. Since microglia play a key role in synaptic maturation and network development of neurons, as well as general homeostasis of cells in the central nervous system, it is important to try and find a solution to the loss of microglia in culture (Liu et al., 2021). It is also worth considering that cells in long term culture could be prone to increased apoptosis, which might lead to pro-inflammatory activation of microglia and associated negative culture conditions. GFAP staining shows a highly ramified morphology for astrocytes in NB and MYM cultures, but interestingly a much higher number of GFAP positive astrocytes are found in MYM cultures compared to NB and NB BP cultures (Fig. 8D, E). Since high GFAP expression is associated with the presence of reactive proinflammatory astrocytes, it is possible that the MYM medium is creating an environment where astrocytes are activated and potentially proinflammatory (Ahtiainen et al., 2021; Byun et al., 2020; Goshi et al., 2020). NMDA and Parvalbumine expression are found colocalized with most if not all NeuN positive neurons in the cultures. This suggests that the neurons in culture can receive signals from neurons using both glutamate and GABA as neurotransmitters, a phenomenon also seen in vivo (Hudson et al., 2020). It would be an interesting follow-up to stain against markers like VGluT and VGAT to try and elucidate the presence of and ratios between neurons using glutamate and GABA as neurotransmitters. This could garner a clear idea of an Excitatory to Inhibitory neuron ratio in the cultures.

For the quantification of neurons, astrocytes, and oligodendrocytes in culture we set up a custom module editor protocol in the MetaXpress software that qualifies a nuclei as positive for these markers based on a combination of marker expression, nuclei size, and intensity compared to local background. To determine if cultures significantly impact the ratios of major cell types as compared to in vivo cortex material, we set up an experiment to stain cultures for these markers. The results were depicted as percentages compared to total nuclei (100%). Literature suggests that adult cortex material (data unclear for embryonic E18 rat cortex material) should contain an estimated 25% of NeuN-positive neurons (Sun et al., 2017), 15-20% Sox9+ astrocytes (Byun et al., 2020; Dos Santos et al., 2020; Keller et al., 2018; Sun et al., 2017) (although this marker is expressed less in adult tissue compared to embryonic tissue), around 20% Olig2+ oligodendrocytes (Keller et al., 2018; Liu et al., 2021; Valério-Gomes et al., 2018), and around 5% Iba-1+ microglia (Dos Santos et al., 2020). These percentages are estimates based on a combination of genetic profiling and immunofluorescence studies in adult rat cortex material. As this literature is based on adult animals, it is difficult to extrapolate the value it has for our embryonic model. At E18 however, all major cell types of the cortex are present in the developing brain, and the results of quantification in adult tissue can serve as a guideline for embryonic development. For our cultures, NeuN positive cell percentages ranged between 15 (NB)

and 25% (NB BP) of total cell percentages (Fig. 6C). The low NeuN percentages for NB cultures can be explained by explosive growth of other cells besides neurons due to high glucose content in the medium. The BP media range between 22-25% of NeuN count, which is nicely in line with literature. Olig2 positive cell percentages range between 18 (MYM) and 25% (NB) for our cultures (Fig. 6D). The MYM and BP cultures (around 22%) are closest to the literature standard of 20%. Sox9 positive cell percentages range between 20-35% for our cultures (Fig. 6E). This is high compared to literature, but Sox9 is expressed in more cells in the developing embryonic cortex compared to adult cortex material where it is only expressed in astrocytes outside of neurogenic regions. Percentages of "Astrocytes," in this study defined Sox9 positive cells minus Sox9+ and Olig2+ double positive cells (which are considered oligodendrocytes), in our cultures range between 15% (MYM and the BP media) to 25% (NB cultures) (Fig. 6F). This is in line with Astrocyte numbers found in literature. Despite these results, it should be stated that this way of quantifying astrocyte numbers is very crude and other options should be explored as a control. Other markers for Astrocytes that can be used to quantify these cells include GFAP (the morphology of this staining makes this tricky, but it can be used to count positive cell bodies, especially when co-stained with a marker like Sox9), ALDH1L1, and S100B. Typically, staining with Sox9, NeuN, and Olig2 leads to a range of 60 (NB) -75% (NC BP) of cells in culture being stained (Fig. 10B), although this is inconsistent between experiments.

Other cell types found in cortex source material include microglia (confirmed, but numbers should be higher than we quantified), smooth muscle cells (vasculature), endothelial cells (vasculature), pericytes, neuronal precursors (NeuN only labels mature post-mitotic neurons), and stem cells (radial glia, neural stem cells) among others. Whether these cells make up the remaining unstained 25-40% of missing cells in these cultures remains to be seen.

To study epilepsy in these cultures, we compared common methods to induce epileptic behaviour in vitro. Specifically, 4-AP addition and the use of a low magnesium recording buffer were compared. 4-AP is a general convulsant drug known to cause epileptic behaviour in neuronal cultures and in vivo (Pacico & Mingorance-Le Meur, 2014). Low magnesium recording buffers cause the threshold for calcium oscillations to be lowered, generally resulting in higher burst frequency, and higher and more consistent peak amplitude (Pacico & Mingorance-Le Meur, 2014). Low magnesium recording buffers reach this effect by removing the competitive inhibition of magnesium on the NMDA receptors that pull calcium into the cell during an action potential (Pacico & Mingorance-Le Meur, 2014). Addition with 33 uM of 4-AP in cultures resulted in a strong convulsant response from all cultures (Fig. 14). The area under the curve was taken as a measure of total activity (all intracellular calcium) and was strongly elevated for all cultures. Comparatively, the use of low magnesium recording buffers showed more tempered results, with notably only the NB cultures showing strong responses (Fig. 15). It results in heightened peak frequency and stabilised peak amplitude and spacing. The lowered impact on cultures grown in MYM and BP medium could be related to those cultures already possessing similar characteristics by default. For a model of epilepsy, the low magnesium condition does create most hallmarks of epileptic behaviour in neuronal cultures. Notably, it stabilizes peak spacing and amplitude. Whilst it does increase peak frequency in cultures grown in NB medium, it does so by lowering the threshold for action potential onset, and not by destabilizing the input of excitatory and inhibitory signals from neurons. Because of these findings, 4-AP addition seems to be a better more physiologically relevant model compared to low magnesium.

To study the involvement of different receptors commonly implied in excitatory and inhibitory neuronal signalling in our cultures, we incubated cells with varying anti-epileptic drugs. Bicuculline is a GABA receptor antagonist. Incubation with this drug causes neurons in culture to produce less frequent bursts with higher amplitude (Pacico & Mingorance-Le Meur, 2014). In our cultures we

supplemented 10 uM of Bicuculline. The NB and MYM cultures responded well to this Bicuculline challenge (Fig. 16). Conversely, the BP cultures showed no effect upon addition at all, with both peak frequency and amplitude remaining unaffected. It is improbable that GABA receptors are not involved in burst formation in these cultures, so higher concentrations should be evaluated. CNQX and MK801 are AMPA and NMDA receptor antagonists, respectively. Inhibition of these glutamate receptors should cause a decrease in peak frequency and amplitude as reported in literature (Pacico & Mingorance-Le Meur, 2014). For NB and MYM cultures, this effect was strong for both antagonists. For the BP cultures both antagonists successfully lowered peak amplitude, but reductions in peak counts were variable and not consistent (Fig. 16). Still, the drastic decreases in average peak amplitude indicate that both receptors are active in these neuronal networks and play a key role in network burst formation. It is an interesting trend that shows that low magnesium epileptic conditions and various anti-epileptic drugs fail to show their full effects in cultures in BP medium conditions. This could be attributed to formation of strong networks that resist effects of these drugs and conditions (which should be assessed using higher concentrations of these AEDs), but it could alternatively indicate that these cultures have neuronal networks that don't fully rely on signalling to these receptors for burst formation. All cultures do clearly indicate glutamate receptors in the onset of network bursting, which confirms the role of excitatory glutamatergic nerve cells in vitro.

To quantify the effects of nutritional interventions on our model we set up a set of experiments to determine if cultures could be significantly modified by nutrient supplementation or removal. To study the change from a carbohydrate-rich to a ketogenic lipid-rich diet, we supplemented cultures with Sodium-3-Hydroxy butyrate in fresh low and high glucose medium (NB cultures only) on DIV 10 and 14 timepoints. On DIV 10, medium changes to fresh high and low glucose medium showed normal performance compared to controls without a medium change. Cultures supplemented with 10 mM of BHB showed a small trend of increasing peak frequency compared to non-supplemented cultures. On DIV 14, all cultured receiving a medium change showed significantly worse calcium oscillations. Especially the low glucose cultures showed almost no remaining activity. Additionally, BHB supplementation showed no positive (or negative) effects on those cultures (Fig. 17). Clearly BHB supplementation by itself has no strong positive effect. It is worth noting that literature suggests that neuronal cultures need a significant amount of time to swap from a carbohydrate-rich diet to a lipid-rich diet (Silva et al., 2022). Because of this, long term culture could allow for a good window for cultures to mature on carbohydrate-rich medium before evaluating the effects of lipid-rich diet on epilepsy onset with a late medium change.

Supplementation of the cultures with a nutritional intervention showed strong effects on network formation and activity. In all cultures supplemented with a nutritional intervention the networks show increased burst count coupled with lower peak amplitude (Fig. 18). Results from staining with Sox9, NeuN, and Olig2 shows that cell count for all cultures is increased when compared to non-supplemented controls. Additionally, nutritional supplementation increased Olig2+ cell count for all conditions compared to controls, and increased astrocyte count for all cultures except NB cultures as compared to non-supplemented controls. NeuN+ neuron count was not significantly or consistently altered, indicating no harmful side effects of the nutritional supplementation for neuronal survival (Fig. 19). The data from these supplementations show clearly that the cultures can be modified in a significant way, and that there is a detectable window for nutritional supplementation studies in our current assays.

Despite these promising results, the culture method has weaknesses that need to be addressed. A combination of long culture duration and high variability in cultures creates a need for high power for assays like the calcium-6 assay. This need for high power coupled with long term culture durations

suggest that the throughput of the model is not high. To increase the suitability of the cultures to model epilepsy in a high throughput manner, validation of the methods with other (functional) assays is a requirement. Multi electrode array recordings can supply much more data regarding burst qualification and onset across the culture and gives an indication of individual neuron cluster contributions. Flow cytometry should also be considered as a means of qualifying and quantifying neuronal subtypes in cultures, as it allows for selection with a wide array of markers.

As it stands the quantification of astrocytes is also a weakness in this protocol. As one of the major cell types in the cortex and the central nervous system, its role is obviously large in ensuring healthy maturation and support of neuronal network formation. Good methods of quantification for these cells are traditionally difficult, but a combination of staining and flow cytometry with multiple markers could give a good estimate. Other cells in the cultures also need to be characterized. The remaining unaccounted-for percentage of cells ranges between 25-40% between culture conditions. It is likely that these cells play a significant role in culture behaviour and seeing how the different culture conditions and supplementations affect the presence and numbers of these cells is crucial in determining the optimal final culture conditions.

To better understand the usefulness of the different epilepsy models it is crucial to study the ability to enhance cultures with nutritional supplements. Seeing how these supplements affect the cultures under epileptic conditions is necessary to determine their value as a disease model. 4-AP shows the most promise of physiologically relevant onset of epileptic behaviour in the neuronal cultures. Supplementing this model with a long-term lipid-rich diet (in the form of ketone bodies like BHB or alternatively with free fatty acids) can be used to study the functioning of the epilepsy model, as well as the ability to alter epileptic behaviour with a ketogenic diet. Additionally, it would be good to evaluate for the effects of anti-epileptic drugs in cultures modelling epilepsy as well. Seeing if these cultures display symptoms of epileptic behaviour can be ameliorated using AEDs is crucial in our validation of the model. For future analysis, we also stored medium at DIV 15/16 from a variety of experiments and cultures to study some components of the conditioned medium (e.g., glucose and ketone bodies levels).

Finally, we have created a good framework for a model of epilepsy in vitro. Based on reliable source material including a variety of different cell types as seen in vivo the model can be optimized to create a realistic, accurate, and sensitive platform in which to evaluate for nutritional supplementations. We have settled on a cell seeding density of 50,000 cells per well, have found LAM to be a stable plate coating, and determined the optimal windows for the application of medium changes to allow for network maturation and suitable time for reconditioning of culture medium. Staining with NeuN, Sox9, and Olig2 to determine the ratios of the major cell types in the cortical cultures leads to reproducible results that suggest that the cultures have good ratios of cells compared to literature. 4-Aminopyridine addition shows good ability to induce epileptic behaviour in all cultures, and most anti-epileptic drugs work to reduce epileptiform behaviour across all cultures. Nutritional supplementation and BHB addition show promising results, but still requires testing and refining to determine a nutritional window for supplementation studies.

Despite all these positive developments, the choice for medium composition is still undecided. All cultures have their strengths and weaknesses that need to be addressed if they are to be used for the culture model. NB cultures suffer from high variability in the calcium-6 assay. Inconsistent peak frequency, spacing, and amplitude make this model potentially unreliable. Additionally, NB cultures show big growth of astrocyte and oligodendrocyte ratios (and percentages) compared to results in literature. This could upset the balance of cells in these cultures and affect the behaviour of the cells in culture. On the other hand, this culture does manage medium changes with grace. It is also

promising to see that long term culture with this medium composition stabilized performance of these cultures in the calcium-6 assay. If these results are reproducible, and show increased performance with medium changes, it could make for an excellent and fully defined model. MYM cultures show much more varied results. These cultures show particularly good results in the calcium-6 assay, with high peak frequency and stable amplitude and peak spacing. Cell numbers are lower, and percentages are more in line with literature compared to NB cultures. It does show big variance in both calcium-6 assay and staining results. At times, this culture protocol produces so many bursts that the quantification software became inaccurate and that the line between noise and signal becomes less clearly interpretable. The BP cultures show particularly good performance in the calcium-6 assay. With stable average-to-high burst formation and very consistent amplitude and spacing for both NB BP and NC BP conditions, these culture methods look strong. Additionally, NB BP and NC BP show cell ratios like literature numbers. NB BP does suffer from loss of activity after medium changes however, making it less suitable for studies involving a medium change to ketogenic conditions. Both models respond well to 4-AP challenges and show a clear window for nutritional supplementation. The major shortcoming for both protocols is that the NC and BP media are undefined and make supplementation difficult for this reason. Analytical detection methods like MS and HPLC could potentially be used to solve these issues and would make NC BP the optimal culturing method with no shortcomings.

The first steps to continue researching and developing this model include repeating the experiments described to confirm the data presented in this report. Continuing to develop and optimize the quantification of the cells in culture with new markers or using different assays like flow cytometry could give a clearer look at the cellular diversity and viability in the cultures. Expanding the characterization efforts to better understand cellular diversity and for example elucidate the role and impact of excitatory and inhibitory neurons could also be very important towards understanding culture behaviour. Pursuing the ability to culture cells long term could open larger windows for network development and maturation, as well as an increased time frame for nutritional intervention and ketosis induction. It is also important to study chronic models of epilepsy, as this is a much more common occurrence in patients with drug-resistant forms of epilepsy. Studying conditioned (and fresh medium in the case of BrainPhys and NeuroCult medium) medium at the end of a culturing period could provide important insight into contents of these media. Specifically, glucose and ketone body quantification could prove invaluable in determining the impact of energy metabolism for network development and activity. After this point, more emphasis should be placed on creating a culturing model that is capable of oscillating under ketogenic conditions. Once a culture model with these dietary conditions is created, it should be combined with a model of epilepsy, and efforts redirected to determining the window for nutritional intervention.

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Supplementary

Medium preparation

Neurobasal medium (NB) without L-glutamine is bought from Gibco. Before use it is supplemented with 2%/1x B27, 1%/1x P/S, and 1%/1x GlutaMAX.

Neurobasal-A medium (NB-A or NB LG (low glucose)) without D-glucose and Sodium pyruvate is bought from Gibco. Before use it is supplemented with 1%/1x Sodium Pyruvate (Gibco, 100x stock, 100 mM stock), 2%/1x B27, and 1%/1x P/S.

NeuroCult neuronal basal plating medium (NC) is bought from Stem Cell Technologies. Before use it is supplemented with 2%/1x SM1 neuronal plating supplement, 1%/1x P/S, and 1%/1x GlutaMAX.

BrainPhys medium (BP) is bought from Stem Cell Technologies. Before use it is supplemented with 2%/1x SM1 neuronal plating supplement, and 1%/1x P/S.

Myelination medium (MYM) is based on DMEM low glucose which is bought from Gibco. Before use, 24.2 mL of DMEM (total volume of MYM is 25 mL) is supplemented with:

- 250 uL or 1x of GlutaMAX.
- 250 uL or 1x of P/S.
- 125 uL of a hormone mix consisting of 1 mg/mL Transferrin, 20 mM of Putrescine, 40 uM of Progesterone, and 6 uM of Sodium selenite.
- 125 uL of 4 ug/mL T3.
- 25 uL of a 100x stock Trace Elements B.
- 6.25 uL of 50 uM Hydrocortisone.
- 5 uL of 50 ug/mL Biotin.
- 5 uL of 1.36 mg/mL Vitamin B12.
- 2.5 uL of 1 mg/mL Ceruloplasmin.
- 12.5 uL of 10 mg/mL Human insulin solution.

After this point the MYM medium is filtered before further addition of:

- 40 uL of 4.2 mg/mL Forskolin.
- 40 uL of 0.1 ug/mL CNTF diluted 1:100 in DMEM low glucose.

Coating preparation

A Poly-D-Lysine Hydrobromide (PDL) stock is created by dissolving 5 mg of PDL in 5 mL of sterile dH2O for a final concentration of 1 mg/mL.

A Natural Mouse Laminin (LAM) stock is created by dissolving Natural Mouse Laminin in DMEM low glucose for a final concentration of 0.5-2 mg/mL.

Supplement preparation

A Sodium-3-Hydroxybutyrate (BHB, Sigma Aldrich) stock solution was made at a concentration of 1M in sterile dH2O.

The nutrient mix used for the nutritional intervention is supplemented directly in medium.

Secondary antibodies

The following secondary antibodies were used in various combinations:

Chicken 488 (For use with primary NeuN, Goat-anti-Chicken, CF-488A, Sigma, SAB4600039, 1:500).

Chicken 594 (For use with primary NeuN, Goat-anti-Chicken, CF-594, Sigma, SAB4600102, 1:500).

Mouse 488 (For use with primary NMDA, Goat-anti-Mouse, CF-488, Invitrogen, A11029, 1:500).

Mouse 647 (For use with primary Olig2, Goat-anti-Mouse, AF647, Life Technologies, A21236, 1:500).

Rabbit 488 (For use with primary Sox9, Iba-1, Goat-anti-Rabbit, CF-488A, Sigma, 4600044, 1:500).

Rabbit 594 (For use with primary Sox9, Goat-anti-Rabbit, CF-594, Sigma, 4600110, 1:500).

Rabbit 647 (For use with primary GFAP, Parvalbumine, Goat-anti-Rabbit, CF-647, Sigma, 4600185, 1:500).

Rat 488 (For use with primary MBP, Goat-anti-Rat, CF-488, Invitrogen, A11006, 1:500).