UNIVERSITY OF CALIFORNIA SAN DIEGO

Developing a Human Cerebral Organoid Epilepsy Model for Studying Live 4D Mitochondrial Dynamics in Response to Ketogenic Diet Treatment

A Thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Biology

by

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The Thesis of Andre Caldas Modolo is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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DEDICATION

I dedicate this thesis to my parents, Lucia and Egidio Modolo, for the unparalleled love and support in every aspect of life, and for always guiding me to become the best version of myself.

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VITA

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ABSTRACT OF THE THESIS

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Approximately 30% of epilepsy patients experience intractable epilepsy, where traditional pharmacological therapies fail to provide seizure relief. Although challenging to sustain, patients often rely on the high-fat, low-carbohydrate ketogenic diet (KD) as a viable alternative for

managing seizures. Despite its clinical success, the exact anti-epileptic mechanisms of the KD remain unclear. Mitochondria have emerged as central players in this mechanism, with studies highlighting them as both targets through which the KD protects against seizures and key regulators of neuronal health and excitability. This research centers on leveraging human cerebral organoids (COs) and CRISPR-Cas9 genome editing to develop an *in vitro* epilepsy model. Utilizing advanced lattice light-sheet microscopy (LLSM), this study captures live 4D (x, y, z, time) morphological and dynamic data of mitochondria in COs, allowing for a comprehensive analysis of mitochondrial network behavior and its response to KD treatment. The study aims to elucidate the mechanisms through which the KD modulates mitochondrial structure and function, potentially guiding the development of novel therapeutics for drug-resistant epilepsy. By integrating cutting-edge *in vitro* human neural tissue models, CRISPR-Cas9 genome editing, and innovative live tissue 4D microscopy techniques, this thesis seeks to advance our understanding of the KD's anti-seizure mechanisms and contribute to the broader field of epilepsy research.

Chapter 1 INTRODUCTION

The Ketogenic Diet, Epilepsy, and Mitochondria

Approximately 30% of epilepsy patients suffer from intractable epilepsy, where traditional pharmacological therapies fail to provide seizure relief (Picot et al., 2008). In such cases, many patients depend on the seizure control induced by the ketogenic diet (KD) (Kossoff et al., 2018). The KD was initially popularized for its anti-seizure effects in the 1920s (Wilder, 1921). Since then, it has been rigorously tested in randomized controlled trials and is now recognized as a valid treatment for drug-resistant epilepsy in clinics worldwide (Freeman et al., 2009; Lambrechts et al., 2017; Neal et al., 2009; Sharma et al., 2013). Despite its consistent use, the exact therapeutic mechanism by which it works to relieve seizures and other epileptic symptoms remains unclear. This diet, usually consisting of a 3:1 or 4:1 ratio of fat to carbohydrate and protein, is implemented to switch the body's primary energy source from glucose to ketone bodies and fatty acids. This strict ratio can be particularly difficult to maintain, frequently leading to patient withdrawal from the treatment (Neal et al., 2009). Consequently, there is significant interest in elucidating the anti-epileptic mechanisms of this diet to aid in the development of novel pharmaceutical therapies for epilepsy cases that currently lack effective drug treatments.

Previous studies exploring the anti-seizure mechanism of the ketogenic diet, and its associated metabolites, suggest that mitochondria play a central role. One school of thought suggests that the ketone bodies—beta-hydroxybutyrate (BHB), acetoacetate (ACA), and acetone—produced in the liver during ketosis directly protect against seizures (Maalouf et al., 2009). One study using a temporal lobe epilepsy mouse model found that BHB administration was enough to mitigate seizure symptoms (Kim et al., 2015). This same study further examined the impact of a ketone body cocktail containing BHB and ACA on mitochondrial state by measuring

mitochondrial permeability transition (MPT) thresholds in mitochondria isolated from the hippocampus of epileptic mice. These thresholds play a crucial role in Ca^{2+} release from the mitochondrial matrix into the cytosol, influence mitochondrial membrane potential, and serve as a good readout of mitochondrial health and function. Treatment with ketone bodies restored the significantly lowered MPT threshold in the impaired mitochondria from epilepsy mice back to healthy wild-type (WT) levels. Furthermore, when drugs were used to lower the MPT threshold, the anti-seizure effects of ketone body treatment were negated (Kim et al., 2015). These findings not only highlight mitochondria as a target through which ketone bodies and the KD exert their anti-seizure effects, but also emphasizes the mitochondria as a central component of neuronal health and excitability. The role of mitochondria in the progression of neurological diseases, such as epilepsy, is well established, making them an especially crucial focus for studying the mechanisms of therapies from altered metabolic pathways.

Mitochondria are heavily implicated in the underlying causes and progression of epilepsy. Mitochondrial DNA (mtDNA) mutations can lead to diseases with epilepsy symptoms and serve as evidence of the involvement of mitochondrial dysfunction in the development of uncontrollable seizures (Frey et al., 2017). One example is a mutation in the mitochondrial tRNA-leu(UUR) gene, which disrupts the translation of electron transport chain (ETC) proteins, compromising ATP production and other mitochondrial functions (Pia & Lui, 2024). This mutation is associated with mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS), a disease in which seizures are commonly seen as symptoms (Fan et al., 2021). Mitochondria are not only tasked with producing 90% of all the body's ATP, but are also heavily involved in crucial cellular functions including the formation and removal of reactive oxygen species (ROS), buffering calcium levels, and coordination of cell death (Friedman & Nunnari, 2014; Osellame et al., 2012).

For proper neuronal function, mitochondria are essential in supplying the copious amounts of ATP needed by ion channels to regulate membrane potential and to modulate neurotransmitter synthesis and release (Duarte et al., 2023). Therefore, dysfunction in mitochondria is often linked with an imbalance in neural circuit firing. Mitochondrial dysfunction not only contributes to seizure symptoms but has also been shown to result from neuronal hyperexcitability, underscoring the critical role of mitochondrial function in the progression of epilepsy. Mitochondrial damage through ROS has been suggested to occur as a result of oxidative stress induced by seizure activity. This has been evidenced by measuring elevated levels of mitochondrial superoxide formation in chemoconvulsant animal models (Liang et al., 2000). One proposed mechanism for the increased levels of ROS during seizure activity is that the mitochondrial oxidative phosphorylation systems become saturated with electrons due to elevated glycolytic activity during intense electrical firing. As a result, excess electrons are leaked and captured by oxygen at an increased rate, leading to higher ROS production (Rowley & Patel, 2013). These ROS then interact with and damage essential mitochondrial components, such as ETC complex I and mtDNA, subsequently leading to a vicious cycle of neural and mitochondrial dysfunction (Jarrett, Liang, et al., 2008; Ryan et al., 2012). Given the mitochondria's central role in neuronal function and excitability, and their vulnerability to seizure-induced damage, investigating how the KD influences mitochondrial function to control seizures is of paramount importance.

Previous studies on the effect of the KD in animal models have shown numerous benefits to mitochondrial health and function, including increased energy production and reduced oxidative stress. One study demonstrated that rats fed a KD for three weeks exhibited an increase in mitochondrial biogenesis and energy reserves in the hippocampus, which correlated with enhanced seizure protection (Bough et al., 2006). Rats fed the KD also had an increase in reduced glutathione

(GSH) levels, a crucial component of the mitochondrial antioxidant pathway (Jarrett, Milder, et al., 2008). Moreover, direct administration of ketone bodies to rats and their brain tissue slices has been shown to be protective and significantly lower levels of mitochondrial ROS, both in response to mitochondrial perturbing drugs and excitotoxicity from elevated levels of neuronal firing (Kim et al., 2007; Maalouf et al., 2007; Maalouf & Rho, 2008). These findings not only associate the KD with rescuing mitochondrial health and neuronal function but highlight the significance of mitochondria as a target for inducing anti-seizure effects. This reinforces the need for further research into the mitochondrial response to this metabolic change. While this research has elucidated the impact of KD treatment on mitochondria in rat models, innovative tissue culture advancements have facilitated the development and examination of human neural tissue samples *in vitro*. This advancement could provide valuable insights into how dietary control of seizures operates within the human cellular environment

In Vitro Human Neural Tissue Epilepsy Model

Cerebral organoids (COs), a three-dimensional *in vitro* model system that recapitulates human brain tissue, can enhance our understanding of how the KD alters human neural physiology. First introduced in 2013 to study the structural brain disorder microcephaly, COs and variations of this model have since exploded in popularity and have been used as a tool to study a variety of neurological diseases (Eichmüller & Knoblich, 2022; Lancaster et al., 2013). These diseases include neurodevelopmental disorders in which epilepsy is an associated symptom such as Timothy syndrome (Birey et al., 2017) and Angelman syndrome (Sun et al., 2019). To generate these organoids, human induced pluripotent stem cells (hiPSCs) are first aggregated into embryoid bodies (EBs) to induce differentiation into the three germ layers - ectoderm, mesoderm, and endoderm. With the aid of a medium formulation that promotes neural identity, differentiation is restricted to the ectoderm, the germ layer that gives rise to neural tissue. Following neural

induction, the neural ectoderm in these EBs self-organize into neural rosettes which have an apicobasal organization similar to the ventricular zone or neural tube *in vivo*. When embedded in a basement membrane matrix (Matrigel), these rosettes receive the structural support needed to develop into a neuroepithelium with three-dimensional spatial organization. Recapitulating *in vivo* brain development, this neuroepithelium generates various types of neural progenitor cells and neurons, including the excitatory and inhibitory neurons essential for studying epilepsy (Lancaster & Knoblich, 2014). These COs are either matured in agitated cultures or sliced and maintained at the air-liquid interface (ALI), where the improved survivability is facilitated by enhanced access to oxygen and nutrients, ensuring better support for the core of the organoid (Giandomenico et al., 2019). ALI-slice cultures can be maintained over extended periods, allowing for the development of more complex neural networks and making them an ideal model for studying long-term dietary perturbations such as the KD. COs offer a neural tissue model that replicates human-specific developmental patterns and intricate neuronal circuits and can have its nutritional environment experimentally manipulated. Moreover, because they are derived from hiPSCs that can have epilepsy-linked mutations introduced, a CO model for epilepsy can be developed, enabling the examination of the mitochondrial responses to KD within the context of human epilepsy.

To bridge the gap between KD research in animal epilepsy models and human-derived disease models, the *Kcna1*-null mouse model for sudden unexpected death in epilepsy serves as an excellent foundation. This model works by knocking out $K_V1.1$, a *Shaker* family voltage-gated potassium channel that is crucial for regulating the duration and frequency of action potentials, thus regulating neuronal excitability (Hille, 1992). With the loss of function of this protein, mice experience frequent spontaneous seizures and other early-onset epileptic phenotypes (Rho et al., 2011; T. A. Simeone et al., 2013; Smart et al., 1998). Treatment of *Kcna1*-null mice with KD has

revealed the KD's efficacy in producing therapeutic effects such as decreasing seizure frequency, normalizing circadian rhythm, and extending lifespan (Fenoglio-Simeone et al., 2009; K. A. Simeone et al., 2016). While these mouse models have been invaluable in our understanding of the KD, the link between mutations in $K_V1.1$ and epilepsy extend beyond animal studies and is frequently observed in human diagnoses as well. This channel is also found in both the human central and peripheral nervous systems and is highly expressed in neurons within the hippocampus, cerebellum, and neocortex (Vacher et al., 2008). Loss-of-function mutations in the human $K_V1.1$ (encoded by the *KCNA1* gene) have been associated with infantile epileptic phenotypes, including cases of drug-resistant seizures (Rogers et al., 2018; Verdura et al., 2020). By employing the guide RNA (gRNA)-mediated DNA endonuclease CRISPR-Cas9 system, hiPSCs can have their *KCNA1* gene specifically targeted and cleaved. (Geyer et al., 2023). In the absence of a donor DNA template, double-stranded breaks can be repaired through non-homologous end joining (NHEJ), a process that may introduce insertions or deletions, leading to loss-of-function mutations. Once a *KCNA1*-null hiPSC line has been established, it can be utilized to generate COs, facilitating the investigation of the KD's effects on human neural physiology with an *in vitro* epilepsy disease model. In addition to evaluating mitochondrial and cellular responses through functional assays that assess markers of oxidative stress, energy production, and MPT, these model systems provide the capability to investigate live subcellular processes by analyzing mitochondrial morphology and behavior.

Live 4D morphology and dynamics as a readout of mitochondrial response to ketogenic diet treatment

To study mitochondrial function *in vitro*, analyzing morphology and dynamics is crucial, as these characteristics are key markers of functional state and cellular condition (Bulthuis et al., 2019). Mitochondrial health and function are intimately linked to their ability to undergo structural

remodeling and transportation processes referred to as mitochondrial dynamics (Giacomello et al., 2020). This includes fusion (the merging of two individual mitochondria into one), fission (the division of a single mitochondrion into two), and transport (either passively through diffusive motion or actively via motor proteins along microtubules). These dynamics are especially essential in highly polarized neurons, which have location-specific mitochondrial requirements, including differing energy demands and calcium regulation needs (Duarte et al., 2023). Most conventional methods of assessing mitochondrial state, such as mitochondrial isolation assays, microscopy of fixed tissue, biochemistry and RNA sequencing, have been crucial for our understanding of the KD's effect on mitochondria. However, there is still a significant gap in methods to visualize live dynamic mitochondrial processes within these model systems. Our lab's lattice light-sheet microscope allows us to collect first-of-its-kind data showing dynamic mitochondrial processes with high frame rate and low phototoxicity, allowing us to image cells in their native tissue environment at the subcellular level for extended periods of time (Chen et al., 2014; Liu et al., 2018). By gathering mitochondrial morphology and dynamics data in four dimensions (x, y, z, and time), we mitigate the information loss inherent in traditional confocal microscopy methods, which typically collect movies on a single plane or capture limited snapshots of the three-dimensional mitochondrial morphology over time. Moreover, with the novel mitochondrial network tracking software MitoTNT, detailed quantitative analysis can be made from not only mitochondrial shape, but its remodeling and motility behavior as well (Wang et al., 2023). Harnessing this innovative microscopy technology with a structure-function approach to study the therapeutic mechanism of the KD may provide deeper insights into how this diet works through the mitochondria to achieve seizure control, resulting in a new avenue for therapeutic intervention.

This thesis will focus on developing a *KCNA1*-null human CO model of epilepsy, establishing KD medium formulations and treatment protocols, and showcasing data from the quantitative analysis of mitochondrial response to KD captured using LLSM. By combining advanced *in vitro* human neural tissue models, hiPSC CRISPR-Cas9 genome editing, and cutting-edge live 4D microscopy techniques, this research aims to enhance our understanding of the mechanisms through which the ketogenic diet modulates mitochondrial function. This work hopes to not only advance our understanding of the KD's therapeutic mechanism but also contribute to the broader field of epilepsy research, potentially guiding the development of more effective treatments for drug-resistant epilepsy.

Chapter 2 MATERIALS AND METHODS

Human induced pluripotent stem cell (hiPSC) culture

All studies involving hiPSCs were performed under approval from the University of California San Diego IRB/ESCRO committee. The WT hiPSC line expressing its translocase of outer mitochondrial membrane 20 (TOM20) protein tagged with a mEGFP was obtained from Allen Institute for Cell Science (Cell line ID AICS-0011 cl.27). hiPSC colonies were grown on tissue culture-treated dishes coated with growth factor-reduced Matrigel (Corning, 354230) in mTeSR Plus (STEMCELL Technologies, 100-0276) using 1% Penicillin-Streptomycin (Thermo Fisher Scientific, 15070063). Cells were dissociated for passaging, electroporation, and embryoid body seeding by first washing with DPBS (Gibco, 14190144) and then lifted using Accutase (STEMCELL Technologies, 07920). Stem cells were routinely checked for pluripotency (Thermo Fisher Scientific, A24881) and tested for *Mycoplasma* using a *Mycoplasma* Detection Kit (abcam, ab289834).

Fluorescent tagging and CRISPR genome editing

The mitochondrial-mEGFP and plasma membrane-mTagRFPT hiPSC line was generated starting with a TOM20-mEGFP hiPSC line. Following protocols detailed in this paper (Oceguera-Yanez et al., 2016), we transfected the AICSDP-42:AAVS1-mTagRFPT-CAAX plasmid (Addgene plasmid # 107580 ; RRID:Addgene_107580) along with gRNA and Cas9 for targeted insertion into the safe harbor locus (AAVS1) in the human PPP1R12C intron. For the *KCNA1*-null line, the double tagged hiPSC line created above was transfected with Cas9 and 3 gRNA targeting three proximal locations in the middle of the human *KCNA1* gene's single exon. The gRNA sequences were: GGAGAUCAAGUUUUACGAGU; GGACGAGGGCUUCAUCAAGG; CGAGAGCUCGGGGCCCGCCA. gRNAs were designed using Synthego Gene Knockout Kit v2.

All transfections were done using the Neon Transfection System (Thermo Fisher Scientific, MPK5000). Colony picking was done after transfection to select for homozygous clones. These clones were analyzed by PCR amplification, gel electrophoresis, and DNA sequencing of the DNA regions containing the cut site. PCR was done with Q5 High-Fidelity 2X Master Mix (New England Biolabs, M0492S). The forward primer targeted 263 bp upstream of the first gRNA cut site (GGATGGCAGCTACCCCCGGCAG). The reverse primer was targeted 221 bp downstream of the third gRNA cute site (CCGTCTTGCTGGGGCAGGCGAA). DNA sequencing was done by Eton Bioscience Inc.

Cerebral Organoid differentiation and culture

Cerebral organoids (CO) were grown from the mitochondria and plasma membrane tagged hiPSC line described above using the STEMdiff Cerebral Organoid Kit (STEMCELL Technologies, 08570). COs were grown and matured according to the kit protocol in kit medium until day 30-40, then embedded in low-melting agarose (Sigma-Aldrich, A9414) and sectioned in cold HBSS buffer (Thermo Fisher Scientific, 14025092) using Leica VT1200 - Semi-Automatic Vibrating Blade Microtome. COs were sliced 300 μm thick using frequencies of 30-60 Hz and speeds of 0.16 mm/s. After slicing, they were placed on 6-well plate cell culture inserts with pore size 0.4 μm (Millicell, PICM0RG50) to be incubated at 37 ℃ for 1-2 hours in serum-supplemented slice culture medium containing DMEM, high glucose with GlutaMAX supplement (Thermo Fisher Scientific, 10566016), 10% FBS (Sigma-Aldrich, F2442), and supplemented with 0.5% glucose (wt/vol) (ThermoFisher Scientific, A16828.0E). After incubation, CO slices were switched to serum-free slice culture medium containing Neurobasal medium (Thermo Fisher Scientific, 21103049), 1X B27 supplement with vitamin A (Thermo Fisher Scientific, 12587010), 1X GlutaMAX supplement (Thermo Fisher Scientific, 35050061), and 25 mM of glucose and fed

three times a week until the KD experiment. Both slice culture mediums were supplemented with 1X Antibiotic-Antimycotic (Anti-Anti) (Thermo Fisher Scientific, 15240062). All CO slices were immobilized using Matrigel on cover glasses for LLSM.

Immunostaining

CO slices were fixed in 4% PFA (Thermo Fisher Scientific, J61899.AK) for 30-minutes at room temperature and then washed three times with PBS. All subsequent steps, including permeabilization and incubation with primary and secondary antibodies, were conducted in a permeabilization buffer (0.5% Triton X-100, 4% normal donkey serum in PBS) at 4°C overnight. The primary antibodies used were anti-MAP2 (1:400; Cell Signaling Technology, 4542S), anti-NeuN (1:200; Invitrogen, 702022), and anti-SOX2 (Invitrogen, MA1-014). The secondary antibodies used were donkey anti-rabbit Alexa Fluor 488 (1:1000; Invitrogen A21206) and donkey anti-mouse Alexa Fluor 568 (1:1000; Invitrogen, A10037). Immunostained images were acquired using an ECHO Revolve fluorescence microscope.

Ketogenic diet treatment

For the 5-day treatment of 50-day old COs, KD medium contained 5 mM β-hydroxybutyrate (BHB) (Thermo Fisher, H6501) and 1 mM glucose (Thermo Fisher Scientific, A24940-01). Ctrl medium contained no BHB and 25 mM of glucose. Both mediums were also composed of DMEM, no glucose(Thermo Fisher Scientific, 11966025), 0.5 mM sodium pyruvate (Thermo Fisher Scientific, 11360070), 1X GlutaMAX supplement (Thermo Fisher Scientific, 35050061), 1X MEM Non-Essential Amino Acids Solution (Thermo Fisher Scientific, 11140050), 1X B27 supplement with vitamin A (Thermo Fisher Scientific, 12587010), 1X N-2 supplement (Thermo Fisher Scientific, 17502048), and 1X Anti-Anti (Thermo Fisher Scientific, 15240062). For the 30-day treatment of 6-month-old COs, KD medium contained 5 mM BHB and 2.5 mM of

glucose, while the control medium had 25 mM of glucose and no BHB. Both media used BrainPhys Neuronal Medium (STEMCELL Technologies, 05790) as the basal medium supplemented with NeuroCult SM1 Neuronal Supplement (STEMCELL Technologies, 05711), 1X GlutaMAX supplement, 1X MEM Non-Essential Amino Acids Solution, 1X N-2 supplement, 5 μM β-Mercaptoethanol, insulin, and 1X Anti-Anti.

Lattice Light Sheet Microscopy

A custom-built lattice light sheet microscope designed by Eric Betzig's Lab at HHMI Janelia/UC Berkeley was used to image samples. Key modifications of this work are the use of 0.6 NA excitation objective lens (Thorlabs, TL20X-MPL), a 1.0 NA detection objective lens (Zeiss W Plan-Apochromat 20×/1.0, model #421452-9800), and a Hamamatsu Photonics Orca Fusion BT sCMOS Camera for image acquisition. 488 nm and 560 nm lasers were used to excite GFP and RFP and/or MitoTracker Green (Thermo Fisher Scientific, M7514) and CellMask Orange (Thermo Fisher Scientific, C10045). A multiple bessel beam light sheet pattern with NA Max 0.4, NA Min 0.35 was used. For the 5-day experiment, a 50 msec exposure time was used per 2D scan and samples were scanned every 300 nm continuously with a piezo-actuated stage. The CO samples imaged were scanned for 201 slices, or 60 microns. For the 30-day experiment, a 25 msec exposure time was used per 2D scan and samples were scanned every 300 nm continuously with a piezo-actuated stage. The CO samples imaged were scanned for 401 slices, or 120 microns. For both experiments, the volumetric frame rates were 10 seconds per frame. Visualizations of mitochondrial data used for the pipeline were done using Imaris (RRID:SCR_007370), ChimeraX (Meng et al., 2023), and Napari (Sofroniew et al., 2024) softwares.

Chapter 3 RESULTS

3.1 Creation of a *KCNA1*-null hiPSC line

The initial hiPSC line featured a green fluorescent protein (mEGFP) tagged to the mitochondrial outer membrane import protein TOM20, and red fluorescent protein (mTagRFPT) tagged to the CAAX domain of K-Ras for plasma membrane localization. This double tagged cell line was developed in our lab from the WTC-11 cell line using resources from the Allen Institute of Cell Science and enables downstream fluorescent imaging. To create a *KCNA1*-null cell line, this double tagged hiPSC line was transfected with CRISPR components, followed by the isolation of a homozygous *KCNA1* knock-out (KO) clone. The transfected CRISPR components consisted of ribonucleoprotein complexes made up of Cas9 and a mixture of three gRNA sequences that target the middle of the coding region of the *KCNA1* gene's single exon. Once transfected, homozygous clones were isolated by sparsely seeding single cells and picking the resulting colonies (pipeline outlined in **Fig. 1A**). Three clones (I, C, and E) had their gRNA-targeted regions PCR-amplified and analyzed by gel electrophoresis to detect any significant insertions or deletions resulting from the NHEJ repair of the CRISPR-Cas9 mediated double stranded DNA breaks. Clones I and C exhibited noticeable deletions in the targeted regions, as evidenced by PCR amplicons appearing slightly lower on the gel compared to the WT hiPSC line. This shift indicates a smaller amplified DNA fragment, with missing base pairs relative to the WT. Clone E appeared to have a heterozygous deletion or was a mosaic clone line with two separate bands, one level with WT and one slightly lower (**Fig. 1B**). The cut site of clone C was sequenced, and after aligning with the WT and reference gene sequences, a series of point mutations followed by an 82 base pair deletion was confirmed after the first gRNA cut site (**Fig. 1C**). Given the high likelihood of this mutation causing a KO in *KCNA1*, clone C was selected to have its protein expression analyzed by western blot. We are still optimizing for detection of this potassium channel protein, due to

challenges such as low abundance and suboptimal antibody detection. Once validated, this cell line will be karyotyped and checked for off-target cutting before being differentiated into COs. With COs made from *KCNA1*-null hiPSCs, further experiments involving measuring calcium activity patterns and depolarization events could be done to validate this as a human *in vitro* epilepsy model.

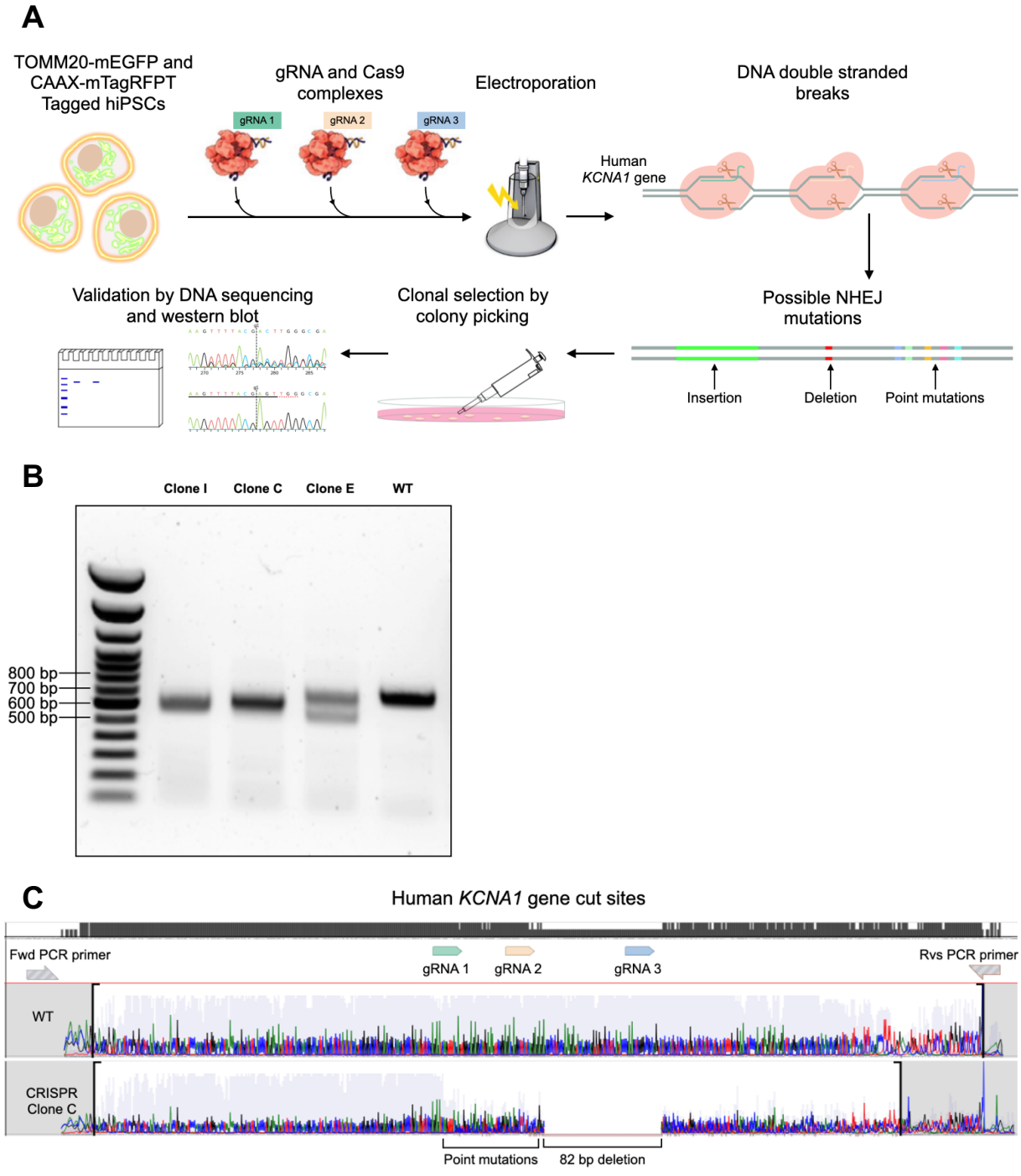

Figure 1. Generation of a *KCNA1*-null hiPSC line via CRISPR-Cas9 genome editing and NHEJ induced mutations. A) Schematic of CRISPR genome editing protocol for targeted Cas9 cutting of the human KCNA1 gene, clone isolation, and KO validation via DNA sequencing and western blot. **B)** Gel electrophoresis analysis of the PCR-amplified DNA region encompassing the three CRISPR cut sites from three isolated clones revealing significant deletions in clone C and I when compared to the WT unedited parental cell line. **C)** Sequencing of the PCR amplicon from clone C reveals point mutations and an 82 bp deletion starting after the gRNA1 cut site, suggesting a KCNA1 KO.

3.2 Generation of WT cerebral organoids as a human neural tissue model for KD treatment and LLSM

In parallel with creating the *KCNA1*-null cell line, WT hiPSCs were used to establish protocols for the generation of human COs, their treatment with KD, and their imaging using LLSM. Utilizing WT cell lines not only supports protocol development but also provides valuable baseline data and insights for mitochondrial behavior. Starting with the double tagged cell line, COs were created through a series of steps: embryoid body formation, neural ectoderm induction, Matrigel-promoted neuroepithelial bud expansion, and agitated maturation (**Fig. 2A**, first row). Once matured to at least 30 days, COs were sliced into 300 μm thick slices using a vibratome and cultured at the air liquid interface (ALI). This slice culture technique minimized internal necrosis by optimizing nutrient diffusion and allowed for better location selection during lattice light-sheet imaging (**Fig. 2A**, second row). COs demonstrated radially organized neural progenitor and stem cells that form an apical basal structure called the neural rosette (**Fig. 2A**, white box and arrow). These formations, similar to the neural tube epithelium *in vivo* brain development, indicate successful neural tissue differentiation. Transcription factor SOX-2 is expressed in the multipotent neural progenitor and stem cells of the neural rosette, and microtubule-associated protein 2 (MAP2) and neuronal nuclei antigen (NeuN) are expressed in neurons that have migrated out to form a cortical plate surrounding the rosette (**Fig. 2B**). Rosette structures were not only used to validate neural tissue differentiation, but also to visually orient and target areas of neuronal circuits within the sample for live lattice light-sheet imaging (**Fig. 2A**, yellow box and arrow). Since KV1.1 channels are expressed in mature neurons of the human hippocampus, cerebellum, and neocortex, targeting areas in the CO where these neurons are present provides the best opportunity to image the cellular populations affected by the *KCNA1* KO and contribute to epilepsy symptoms.

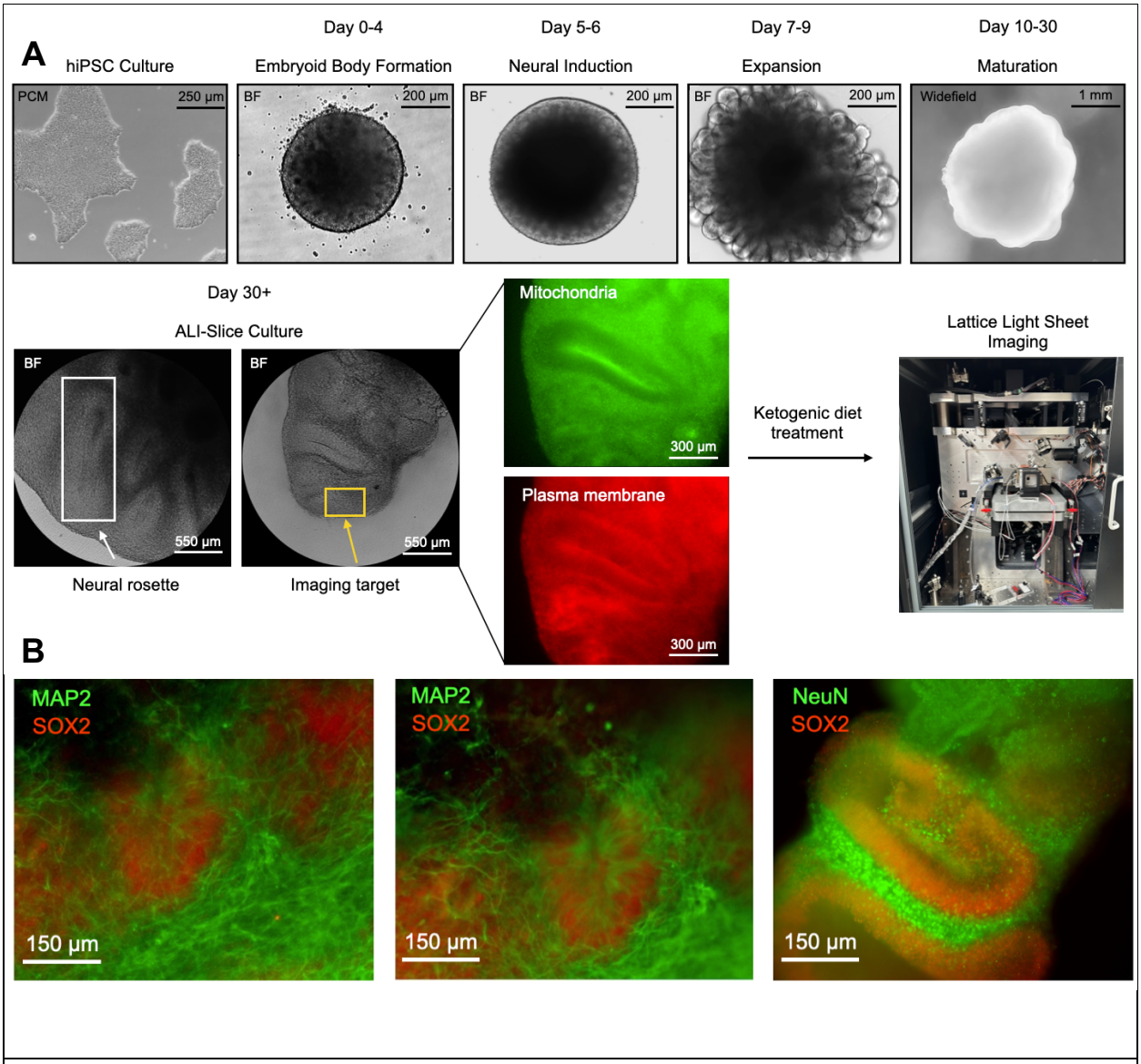

Figure 2. Development of WT cerebral organoids, their treatment with KD medium, and subsequent live imaging using LLSM. A) Pipeline for KD experimentation with hiPSC-derived CO samples, encompassing the differentiation of COs, ALI-slice culture, KD medium treatment, and imaging with LLSM. Neural rosette formations are shown by white boxes and arrows, and imaging targets where mature neurons are deposited are shown in yellow boxes and arrows. **B)** Immunostaining of MAP2 and NeuN (neuron markers) and SOX2 (neuronal progenitor and stem cell markers) in 40-day old COs shows multipotent cell types in rosettes and neurons in the surrounding region.

Two rounds of experiments involving KD treatment of WT CO slices were conducted. In the first experiment, 50-day old organoids were treated with KD medium for 5 days. Neurons typically begin to appear around the neural rosettes after approximately 20 days of maturation (Eichmüller & Knoblich, 2022). In the second round, the age and treatment period were extended to 180 and 30 days respectively to assess whether more mature and complex neural circuits, along with a longer KD exposure, would yield more pronounced effects. The control (Ctrl) and KD medium formulations were adjusted between the 5-day and 30-day experiments to include BrainPhys neuronal culture medium as the basal medium, supplemented with insulin and β-mercaptoethanol (see **Table 1.** and **Table 2.** for medium compositions). These changes were made to better promote neuronal health and maturation over the extended treatment period (Bardy et al., 2015). The neural growth medium compositions for Ctrl and KD only differed in their fuel source concentrations. The Ctrl medium contained 25 mM of glucose for both experiments, and KD medium contained 1 mM of glucose for the 5-day treatment, and 2.5 mM of glucose for the 30-day treatment. Glucose level for the Ctrl medium was in agreement with that of the medium formulations in the paper establishing the ALI CO slice culture method (Giandomenico et al., 2019). KD medium included 5 mM of β-hydroxybutyrate (BHB), the most abundant and stable ketone body used for energy production in humans (Newman & Verdin, 2017). KD medium was designed to approximate the nutrient levels that would be present in children undergoing ketosis. Previous studies indicate that patients on a clinically administered KD have blood plasma levels of BHB around 4-5 mM and that 4-6 mM of BHB is suggested to be neuroprotective (Fedorovich et al., 2018; Neal et al., 2009). Nutrient concentrations resulted in a glucose ketone index (GKI) that meets the therapeutic ketosis criterion of being less than or equal to 1 (Meidenbauer et al., 2015). Once treated with KD medium, CO samples were immobilized on glass coverslips using

Matrigel and were ready to be imaged live on our lattice light-sheet microscope. For the 30-day treatment, COs were also stained with MitoTracker Green FM and CellMask Orange Plasma Membrane Stain for better signal during imaging.

Acknowledgements

The culture of COs and the design of the KD medium were carried out with the assistance of postdoctoral researcher Marta Medina Carbonero.

Table 1. Medium formulations for 5-day treatment

Control Medium		Ketogenic Medium	
Ingredient	Concentration	Ingredient	Concentration
DMEM/F12 (no glucose, no L-glutamine, no sodium pyruvate, no phenol red, with HEPES)	Base	DMEM/F12 (no glucose, no L-glutamine, no sodium pyruvate, no phenol red, with HEPES)	Base
Glucose	25 mM	Glucose	1 mM
β-hydroxybutyrate (BHB)	0 mM	β-hydroxybutyrate (BHB)	5 mM
Sodium pyruvate	0.5 mM	Sodium pyruvate	0.5 mM
GlutaMAX (100X)	1X	GlutaMAX (100X)	1X
MEM non-essential amino acids (100X)	1X	MEM non-essential amino acids (100X)	1X
B27 (50X)	1X	B27 (50X)	1X
N2 (100X)	1X	N2 (100X)	1X
Anti-Anti (100X)	1X	Anti-Anti (100X)	1X

Table 2. Medium formulations for 30-day treatment

Control Medium		Ketogenic Medium	
Ingredient	Concentration	Ingredient	Concentration
BrainPhys Neuronal Medium	Base	BrainPhys Neuronal Medium	Base
Glucose	25 mM	Glucose	2.5 mM
β-hydroxybutyrate (BHB)	0 mM	β-hydroxybutyrate (BHB)	5 mM
Sodium pyruvate	0.5 mM	Sodium pyruvate	0.5 mM
GlutaMAX (100X)	1X	GlutaMAX (100X)	1X
MEM non-essential amino acids (100X)	1X	MEM non-essential amino acids (100X)	1X
B27 (50X)	1X	B27 (50X)	1X
N2 (100X)	1X	N2 (100X)	1X
β-mercaptoethanol	5 µM	β-mercaptoethanol	5 µM
Insulin	2.87 µg/ml	Insulin	2.87 µg/ml
Anti-Anti (100X)	1X	Anti-Anti (100X)	1X

3.3 Live 4D microscopy of KD-treated cerebral organoids reveals significant shifts in mitochondrial behavior that depends on treatment duration

For each medium condition, two CO samples were imaged at three to four different locations. 3D movies of the mitochondria from these neuronal regions were captured at a time resolution of 10 seconds per volumetric frame. For each location on a sample, live imaging was done for 10 minutes capturing a total of 60 volumetric frames. For the 5-day experiment, a 60 by 170 by 30 μm volume was imaged, and for the 30-day experiment, a slightly larger 120 by 170 by 30 μm volume was captured. These ten-minute 3D mitochondria movies were processed via an optimized deconvolution, deskew, and rotation pipeline developed in our lab (detailed pipeline in our most recent paper: Wang et al., 2024). The processed data was divided into smaller regions of interest and analyzed using MitoTNT, a mitochondrial temporal network tracking software developed in our lab. This software tracks and quantifies mitochondrial morphological, network remodeling, and motility data (Wang et al., 2023). Mitograph (Viana et al., 2015) segments the mitochondria and skeletonizes each piece, then MitoTNT uses spatial proximity and network topology to compute the optimal frame to frame tracking (**Fig. 3A**). This analysis not only allows for morphological assessments such as measurement of fragment and branch length, but also quantification of dynamic processes such as motility and fission and fusion rates.

Morphological measurements are derived from mitochondria skeletons by calculating both the length of the entire piece (fragment length) and the length of branching pieces connected to a fragment (branch length). After 5 days of KD medium treatment, 55-day-old CO mitochondria showed elongation in their morphology (**Fig. 3B**) exhibiting a 40% increase in their average fragment length and a 19% increase in their average branch length compared to those treated with

Ctrl medium (**Fig. 3D**). After the 30-day treatment of 180-day-old COs, the same trends were observed: the KD-treated group exhibited a visibly more hyperfused and networked morphology, while the Ctrl group showed a fragmented morphology (**Fig. 3C**). The 30-day KD-treated COs exhibited a more substantial increase in average fragment length compared to the 5-day KD-treated COs, with a 70% increase compared to its Ctrl group (**Fig. 3D**). Analyzing the mean of the top 100 largest fragments in both KD-treated groups, the 30-day treatment resulted in an average fragment length of 34 μm, while the 5-day treatment averaged only 12 μm. These findings suggest that longer treatment periods are crucial in capturing the full extent of the mitochondrial elongation in response to KD treatment. Another interesting observation is that mitochondria within neuron cell bodies displayed a punctate or fragmented morphology, regardless of the treatment condition (see circles in **Fig. 3C**). This supports the idea that changes in mitochondrial morphology may be regulated according to local bioenergetic or functional needs known to be variable within highly polarized neurons.

To further investigate the change in mitochondrial behavior, we quantified the levels of fission and fusion using MitoTNT to analyze remodeling rates. Fusion events were quantified by tracking the merging of two mitochondrial nodes (nodes are points spaced at 220 nm along the mitochondrial fragment), while fission events were counted by tracking the separation of two nodes. These events were only considered if the remodeling persisted (connected or divided) for at least 5 frames after occurring. For the 5-day treatment, average fusion counts did not significantly change between the two medium conditions, while average fission counts were increased by 19% in the KD medium group (**Fig. 3E**). In the 30-day treatment, both the average fission and fusion counts were increased in the KD group, by 57% and 21% respectively (**Fig. 3E**). This data could suggest an overall increase in mitochondrial remodeling activity while in ketogenic

conditions. Because mitochondrial turnover, which is governed by fission and fusion processes, has been shown to be on the order of 10-30 days depending on the tissue type, fluctuations in this ratio may be occurring at a time scale outside of the 10-minute imaging window (Beattie et al., 1967; Kowald & Kirkwood, 2011; Markaki & Tavernarakis, 2020). Therefore, to delve deeper into mitochondrial dynamics, the motility of individual mitochondrial fragments was assessed to determine if this diet induces more hyperactive mitochondrial movement and reorganization.

Mitochondrial motility measurements were determined by calculating the diffusion coefficients from the trajectory of the tracked fragments, branches, and nodes. After the 5-day KD treatment, COs had mitochondria with average fragment, branch, and node diffusivities that were 27%, 28%, and 34% higher, respectively, compared to those treated with the Ctrl diet (**Fig. 3F**). In the 30-day experiment, this trend was even more pronounced, with the KD-treated group showing increases in average fragment, branch, and node diffusivities of 101%, 76%, and 74%, respectively (**Fig. 3F**). These results not only suggest that ketogenic conditions may induce more hyperactive mitochondrial motility but also reinforce the observation that longer treatment durations or older CO ages are necessary to fully realize the extent of mitochondrial behavioral responses to the new metabolic environment.

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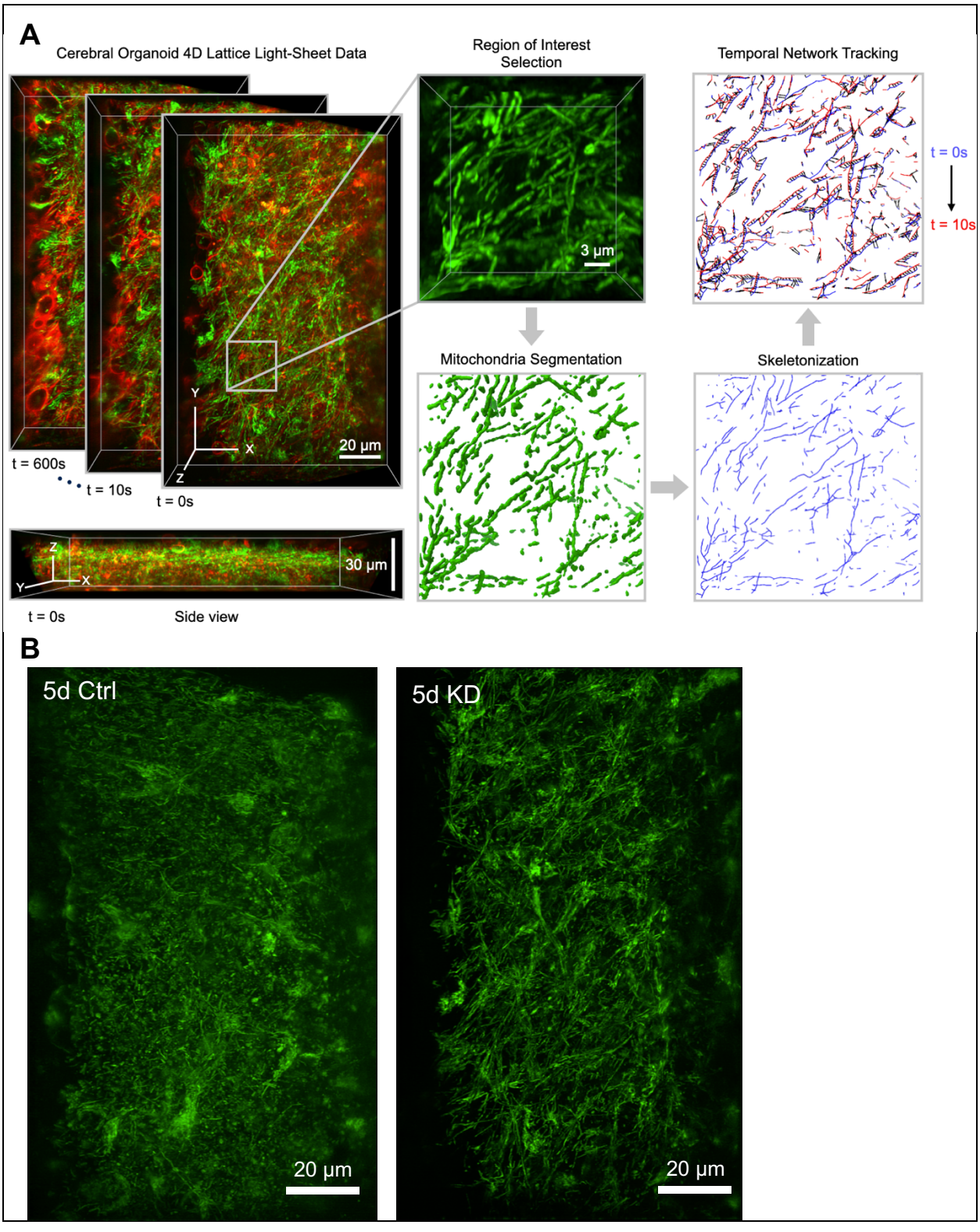
Figure 3. Skeletonization and temporal network tracking of lattice light-sheet data reveal shifts in mitochondrial morphology and dynamics within COs that become increasingly pronounced with the duration of KD treatment. A) A schematic is shown of the MitoTNT pipeline starting with a 10 minute, 60 frame movie of a 60 by 170 by 30 μm volume of neurons from the CO slice. **B-C)** A single 3D frame of the CO mitochondria in the Ctrl group (left) and KD group (right) after the 5-day treatment **(B)** and 30-day treatment **(C)**. **D-F)** Bar plots of MitoTNT measurements are shown with the x-axis representing the treatment conditions and the gray bar representing a 99% confidence interval. **D)** Average fragment and branch lengths for the 5-day and 30-day treatments are plotted with the y-axis indicating the lengths of the skeletonized mitochondria in microns. In the 5-day KD treatment, mitochondria exhibited a 40% increase in average fragment length compared to those treated with Crl, while the 30-day treatment resulted in a more substantial elongation, with a 70% increase in the same measurement. **E)** Average fusion and fission count for the 5-day and 30-day treatments are plotted with the y-axis representing the number of times, during the 10-minute imaging, that a mitochondrial node fused or underwent fission normalized per 1000 mitochondrial nodes. These measurements reveal a general increase in remodeling activity in the mitochondria treated with KD, which escalated with longer treatment periods. **F)** Average diffusivity values for mitochondria at the fragment, branch and node levels for the 5-day and 30-day treatments are shown with the y-axis indicating the diffusivity coefficient of the skeletonized mitochondria in μm^2/ second. After a 5-day KD treatment, mitochondria fragment, branch, and node motility were increased 27%, 28%, and 34% respectively compared to control. The 30-day KD treatment demonstrated a more pronounced increase in mitochondrial movement, with the KD-treated group showing a 101% increase in fragment diffusivity, a 76% increase in branch diffusivity, and a 74% increase in node diffusivity compared to the control. An independent student t-test was performed for all measurements. $p < 0.0001$ (* * * *).


A
Cerebral Organoid 4D Lattice Light-Sheet Data
Region of Interest Selection
Temporal Network Tracking
t = 0s
t = 10s
3 µm
20 µm
t = 600s
t = 10s
t = 0s
Mitochondria Segmentation
Skeletonization
30 µm
t = 0s
Side view
B
5d Ctrl
5d KD
20 µm
20 µm

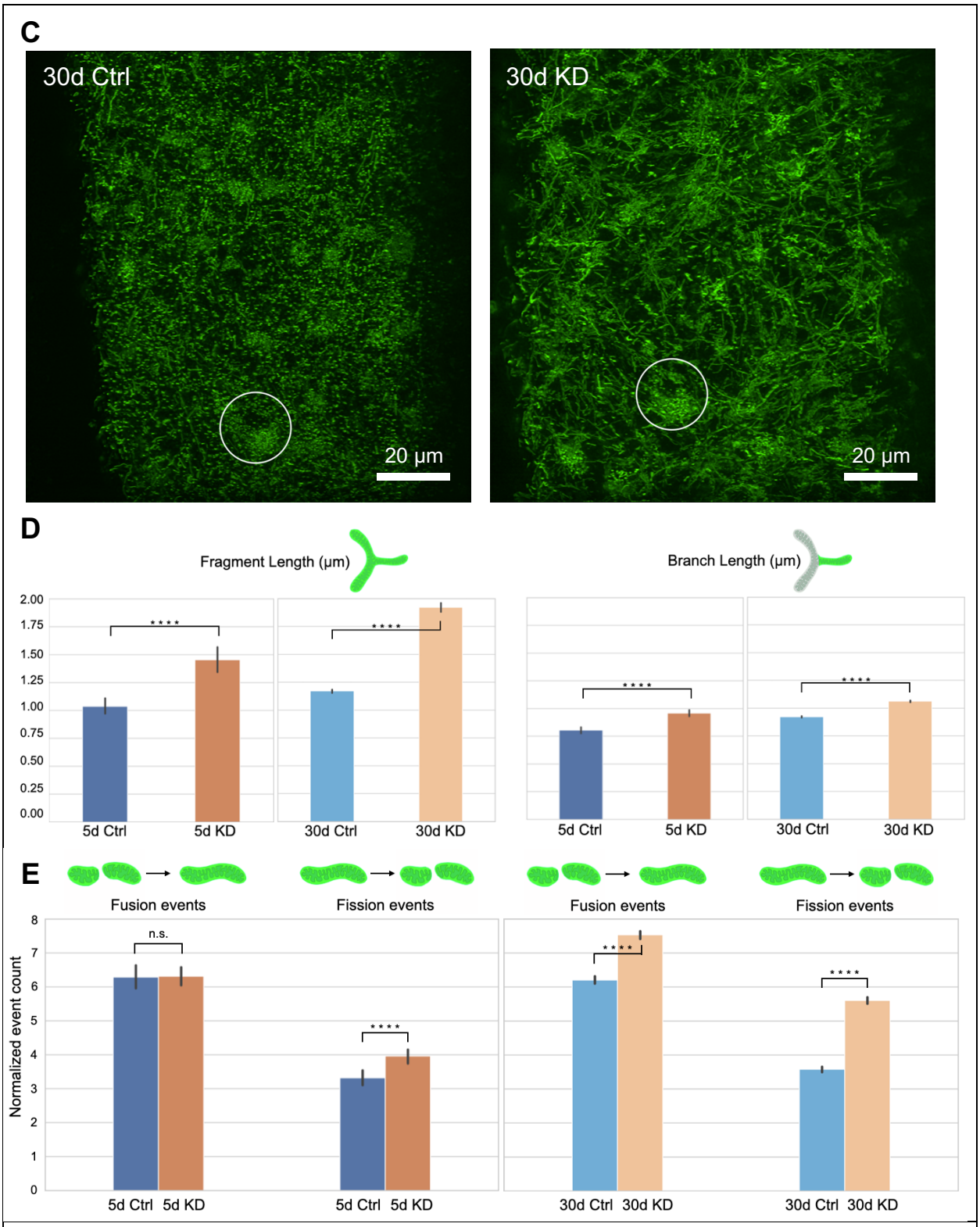


Figure 3. Skeletonization and temporal network tracking of lattice light-sheet data reveal shifts in mitochondrial morphology and dynamics within COs that become increasingly pronounced with the duration of KD treatment. Continued…

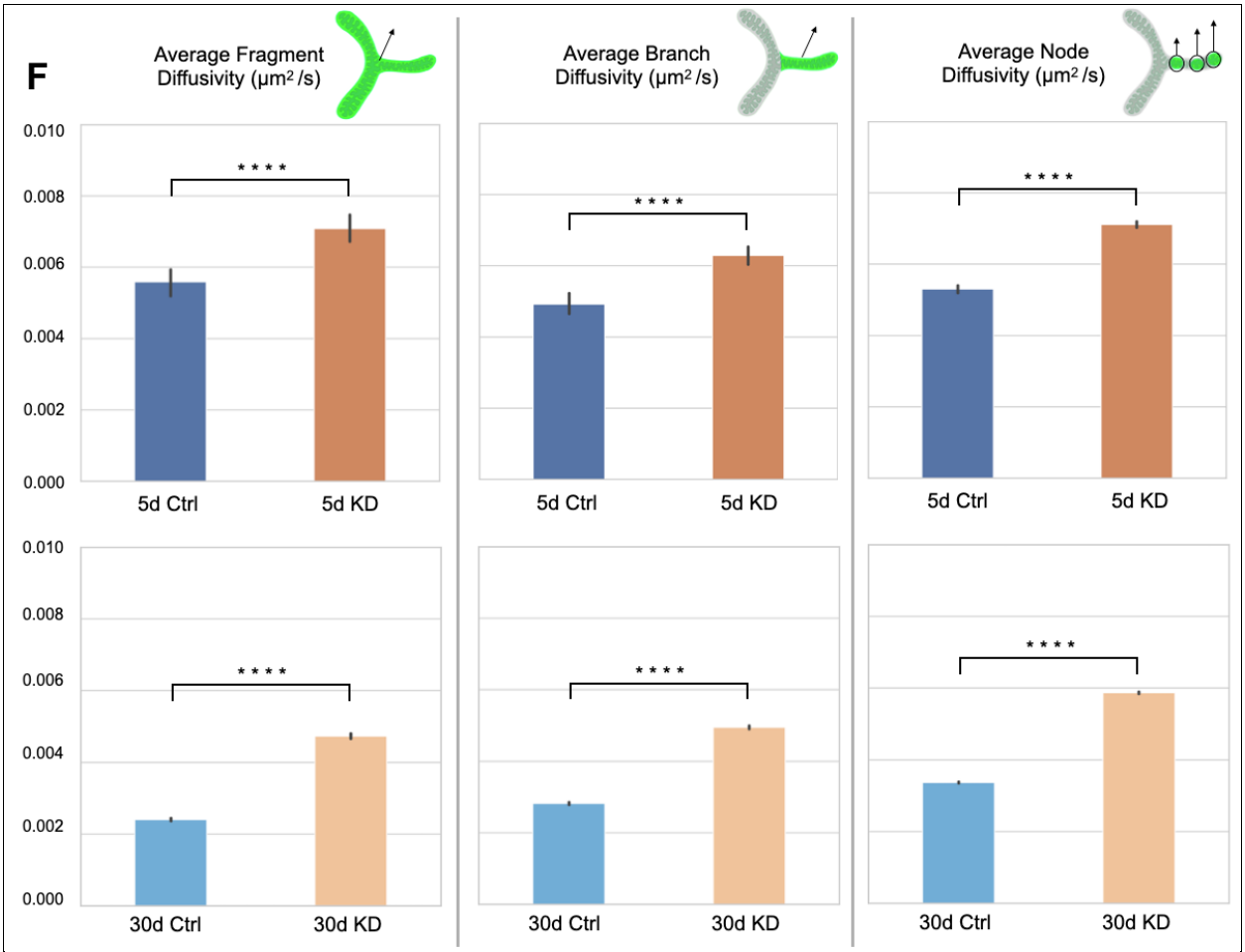


Figure 3. Skeletonization and temporal network tracking of lattice light-sheet data reveal shifts in mitochondrial morphology and dynamics within COs that become increasingly pronounced with the duration of KD treatment. Continued…

DISCUSSION

This thesis work leverages CRISPR genome editing of hiPSCs, CO differentiation, and LLSM to develop an *in vitro* epilepsy model that enables the study of live tissue mitochondrial dynamics in response to KD treatment. While the validation of the hiPSC line with an epilepsy-linked *KCNA1*-null mutation is ongoing, protocols for CO culture, KD treatment, and sample imaging have been successfully established, allowing valuable data to be obtained from WT samples. From the morphological measurements, KD treatment is seen to significantly elongate or hyperfuse the mitochondria in these neural tissue samples. These general morphological observations are in line with previous studies noting fragmented mitochondria in nutrient-rich environments and elongated mitochondria in starvation or low nutrient models (Gomes et al., 2011; Molina et al., 2009). Moreover, elongated mitochondria have also been observed in brain tissue slices of autism mouse models after their symptoms were alleviated through KD treatment (Ahn et al., 2020). Mitochondrial elongation and hyperfusion have been shown to provide several beneficial effects, such as optimizing ATP production, rescuing damaged or mutated mitochondria through a component-sharing process known as complementation, and protecting against apoptosis (Liesa & Shirihai, 2013; Tondera et al., 2009; Rambold et al., 2011; Gomes et al., 2011). These cellular benefits are similar to those induced by the ketogenic diet, potentially linking mitochondrial hyperfusion to the diet's anti-seizure mechanism.

Measuring fission and fusion rates as well as tracking mitochondrial motility has suggested that KD treatment induces more hyperactive behavior. Few studies have explored this type of mitochondrial behavioral response to low glucose and/or ketone body exposure, primarily due to the lack of necessary technology. Recent cutting-edge advancements in tracking subcellular

components over 3D space and time enable these studies. The observed increases in mitochondrial motility and reorganization may result from enhanced active transport or crosstalk with other organelles, such as the ER. Studying organelles known to commonly interact with mitochondria would provide valuable context for understanding changes in their movement activity.

A notable trend observed in the morphological, remodeling, and motility data is that the 30-day treatment amplified the effects seen with the 5-day treatment. Mitochondria in the 30-day treatment group exhibited an even more elongated morphology and more significant increases dynamic measurements compared to their respective controls. The time dependent mitochondrial response to KD treatment has been observed before in a study looking at a MELAS neuronal cybrid cell model. The mitochondria from these cells only displayed significant improvement in respiration and complex I activity after 4 weeks of KD treatment (Frey et al., 2017). Moreover, clinical studies on the efficacy of the KD in refractory epilepsy patients typically observe improvements in seizure control over a 1–3-month period, indicating that acute implementation of this diet is insufficient to elicit its full anti-seizure effects (D'Andrea Meira et al., 2019). The similar timeframe for the onset of anti-seizure effects, improved mitochondrial oxidative metabolism, and increased elongation and hyperactivity of mitochondria suggests that a switch in mitochondrial morphology and dynamics may be crucial for enhancing mitochondrial function and its downstream effects on neuronal excitability. While these results show a promising start for developing new models and readouts of live responses to KD treatment in human tissue, more experiments and replicates are required to fully validate these observations. Differences between the measurements in the 5-day and 30-day treatments could be due to differences in CO age and thus neuronal circuit maturity and complexity, as well as differences in basal medium. Future experiments will involve controlling for CO age, batch to batch differences, medium formulations,

and imaging conditions while capturing mitochondrial data from validated *KCNA1*-null COs. These findings lay a robust foundation for further research into the mitochondrial response to KD treatment in human tissue, with future studies needed to refine the model and explore its potential. A more comprehensive understanding of the role of mitochondria in the KD's anti-seizure mechanism will lay the groundwork for developing drugs that emulate this mitochondrial behavior, potentially yielding novel therapeutics for drug-resistant epilepsy.

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