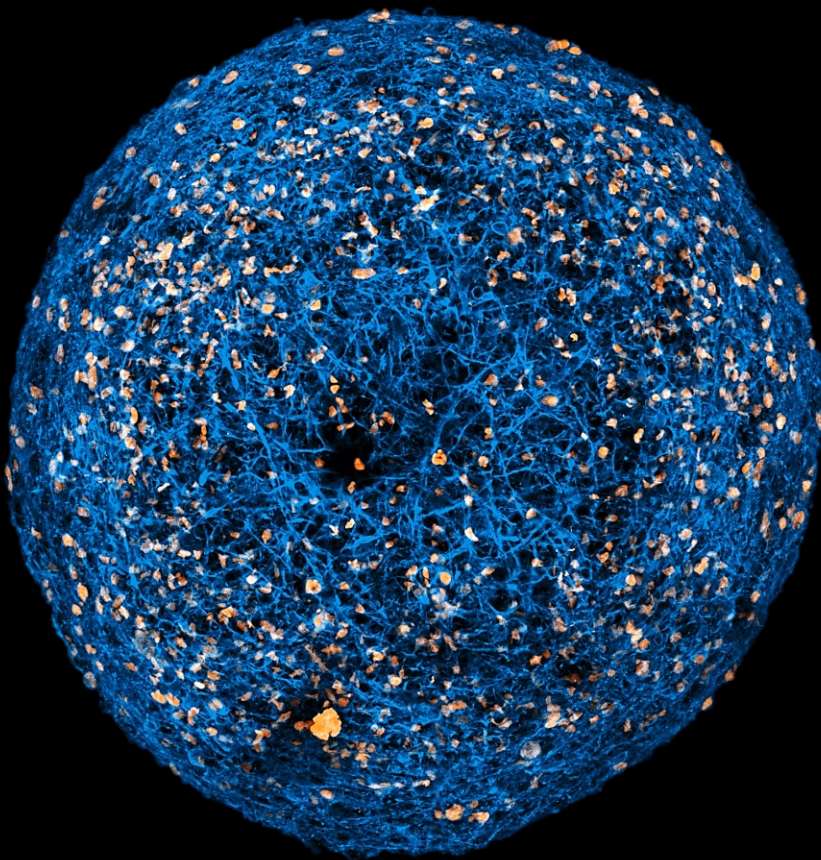


Dissecting neuronal mitochondrial disorders with human pluripotent stem cell-derived neurons and brain organoids

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Dissecting neuronal mitochondrial disorders with human pluripotent stem cell-derived neurons and brain organoids

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Summary

Mitochondrial dysfunction is a hallmark of neurodegenerative diseases such as Leigh syndrome (LS) and Huntington's disease (HD). The inherent species-specific mechanisms driving the human pathology hinder the discovery of disease-modifying therapies using animal models. Human induced pluripotent stem cells (iPSC) technology facilitates disease modeling by enabling patient-specific studies of human neuronal pathology.

Emerging evidence suggests that mitochondrial dynamics and bioenergetics are key drivers of neuronal morphogenesis, an event that is disrupted in LS and HD patient-derived neural models. Here, I used iPSC-derived 2D neurons and midbrain organoids to investigate mechanistic insights into HD pathology and to explore neuronal morphogenesis as a molecular target for drug discovery in LS.

In the context of HD, I unveiled how mutant huntingtin (mHTT) impacts midbrain organoid development by promoting early neuronal fate commitment and reducing the pool of neuronal progenitor cells (NPCs). Transcriptomic studies highlighted the dysregulation of CHCHD2 (coiled-coil-helix-coiled-coil-helix domain containing 2), a transcription factor involved in mitochondrial integrated stress response whose altered expression was associated with abnormal mitochondrial morpho-dynamics. This project uncovered how mHTT disrupts human neurodevelopment by altering neurometabolic programming *via* dysregulation of CHCHD2.

In LS, mutations in *SURF1*, an assembly factor of respiratory complex IV of the mitochondrial electron transport chain, lead to defective neurometabolic programming of NPCs, resulting in inefficient neurite outgrowth. Here, I developed a high-content imaging pipeline to measure neuronal morphogenesis to explore the potential of this phenotype for compound discovery. In combination with *in silico* and yeast *in vitro* compound screens, I identified two compounds that pharmacologically rescued neuromorphogenesis in iPSC-derived induced neurons: Talarozole and Sertaconazole.

I furthermore validated the efficacy of these compounds in reversing key disease-specific features in patient-derived midbrain organoid models of LS. Both compounds reduced organoid size, diminished the excess of lactate released into the media, and ameliorated the overall neuronal arborization. Talarozole and Sertaconazole showed similar chemical structures and docking features, suggesting pleiotropic effects with certain similarities in their mode-of-action.

Altogether, this study highlights the use of human iPSC-derived neuronal models to identify key molecular mechanisms driving disease pathology in neurodevelopmental alterations. Beyond disease modeling, my work underscores the potential of these models as drug discovery platforms for rare diseases like LS, for which animal models have failed to recapitulate the complexity of the human disease. This work unveiled two potential drug compounds for the treatment of LS and opened new avenues of investigation of new molecular targets to develop effective treatments.

Zusammenfassung

Mitochondriale Dysfunktion ist ein Kennzeichen neurodegenerativer Erkrankungen wie z.B. des Leigh-Syndroms (LS) und der Huntington-Krankheit (HD). Der artspezifische Mechanismus, der der menschlichen Pathologie zugrunde liegt, erschwert die Entdeckung krankheitsmodifizierender Therapien anhand von Tiermodellen. Die Technologie der humanen induzierten pluripotenten Stammzellen (iPSC) erleichtert die Modellierung von Krankheiten, indem sie patientenspezifische Studien der menschlichen neuronalen Pathologie ermöglicht.

Neue Erkenntnisse deuten darauf hin, dass die mitochondriale Dynamik und Bioenergetik die neuronale Morphogenese entscheidend beeinflussen - ein Vorgang, der in neuronalen Modellen von LS- und HD-Patienten gestört ist. Hier verwende ich von iPSC-abgeleitete 2D-Neuronen und Organoide des Mittelhirns, um mechanistische Einblicke in die Pathologie der Huntington-Krankheit zu gewinnen und die neuronale Morphogenese als molekulares Ziel für die Entdeckung von Medikamenten bei LS zu untersuchen.

Im Zusammenhang mit der Huntington-Krankheit habe ich herausgefunden, wie die Huntingtin-Mutante (mHTT) die Entwicklung von Mittelhirn-Organoiden beeinflusst, indem es die frühe neuronale Differenzierung fördert und den Pool an neuronalen Vorläuferzellen (NPCs) reduziert. Transkriptomische Studien haben die Dysregulation von CHCHD2 (coiled-coil-helix-coiled-coil-helix domain containing 2) aufgezeigt, einem Transkriptionsfaktor, der an der integrierten mitochondrialen Stressantwort beteiligt ist und dessen veränderte Expression mit einer abnormalen mitochondrialen Morphodynamik in Verbindung gebracht wurde. In diesem Projekt wurde aufgedeckt, wie mHTT die menschliche Neuroentwicklung stört, indem es die neurometabolische Programmierung über die Dysregulation von CHCHD2 verändert.

In LS führen Mutationen in SURF1, einem Assemblierungsfaktor des Atmungskomplexes IV der mitochondrialen Elektronentransportkette, zu einer fehlerhaften neurometabolischen Programmierung von NPCs, was zu einem ineffizienten Neuritenwachstum führt. Hier habe ich eine High-Content-Imaging-Pipeline zur Messung der neuronalen Morphogenese entwickelt, um das Potenzial dieses Phänotyps für die Entdeckung von Wirkstoffen zu untersuchen. In Kombination mit *In-silico*- und *In-vitro*-Screens von Hefewirkstoffen habe ich zwei Wirkstoffe identifiziert, die die Neuromorphogenese in iPSC-abgeleiteten induzierten Neuronen pharmakologisch gerettet haben: Talarozol und Sertaconazol.

Darüber hinaus habe ich die Wirksamkeit dieser Wirkstoffe bei der Umkehrung wichtiger krankheitsspezifischer Merkmale in von Patienten abgeleiteten Organoidmodellen des Mittelhirns von LS validiert. Beide Substanzen verringerten die Größe der Organoide, sowie den Überschuss an Laktat, der in das Medium abgegeben wird, und verbesserten die allgemeine neuronale Verzweigung. Talarozol und Sertaconazol wiesen ähnliche chemische Strukturen und Docking-Merkmale auf, was auf pleiotrope Effekte mit gewissen Ähnlichkeiten in ihrer Wirkungsweise schließen lässt.

Insgesamt unterstreicht diese Studie die Verwendung humaner von iPSC-abgeleiteter neuronaler Modelle zur Identifizierung molekularer Schlüsselmechanismen, die die Krankheitspathologie bei neuroentwicklungsbezogenen Veränderungen bestimmen. Über die Modellierung von Krankheiten hinaus unterstreicht meine Arbeit das Potenzial dieser Modelle als Plattformen für die Entdeckung von Medikamenten für seltene Krankheiten wie LS, für die Tiermodelle die Komplexität der menschlichen Krankheit nicht wiedergeben können. Diese Arbeit brachte zwei potenzielle Wirkstoffe für die Behandlung von LS ans Licht und eröffnete neue Wege zur Erforschung neuer molekularer Ziele für die Entwicklung wirksamer Behandlungen.

Abbreviations

2-DG	2-Deoxyglucose	DRP1	Dynamin-Related Protein 1
3-NP	3-Nitropropionic Acid	EMA	European Medicines Agency
AAV	Adeno-Associated Virus	ETC	Electron Transport Chain
Acetyl-CoA	Acetyl Coenzyme A	F-actin	Filamentous Actin
ACSF	Artificial Cerebrospinal Fluid	FADH2	Flavin Adenine Dinucleotide (Reduced)
AD	Alzheimer's Disease	FDA	Food and Drug Administration
ADP	Adenosine Diphosphate	GPI	Globus Pallidus Internus
AMP	Adenosine Monophosphate	GPe	Globus Pallidus Externus
AMPK	AMP-Activated Protein Kinase	GRP75	Glucose-Regulated Protein 75
ATP	Adenosine Triphosphate	HCA	High Content Analysis
ASD	Autism Spectrum Disorder	HCI	High Content Imaging
BMP	Bone Morphogenetic Protein	HD	Huntington's Disease
Ca ²⁺	Calcium Ion	HIF1 α	Hypoxia-Inducible Factor 1 Alpha
CBD	Cannabidiol	HTT	Huntingtin
CHCHD2	Coiled-Coil-Helix-Coiled-Coil-Helix Domain Containing 2	IGF-1	Insulin-Like Growth Factor 1
CNS	Central Nervous System	IMM	Inner Mitochondrial Membrane
COX	Cytochrome C Oxidase	iNs	Induced Neurons
COX15	Cytochrome C Oxidase Subunit 15	iPSC	Induced Pluripotent Stem Cells
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats	KD	Knockdown
CYP26A/B1	Cytochrome P450 Family 26 Subfamily A/B1	KIF5	Kinesin Family Member 5
CYP51A1	Cytochrome P450 Family 51 Subfamily A1	KLF4	Krüppel-Like Factor 4
D1	Dopamine Receptor D1	LC3	Microtubule-Associated Protein 1A/1B-Light Chain 3
D2	Dopamine Receptor D2	LHON	Leber's Hereditary Optic Neuropathy
DAPI	4',6-Diamidino-2-Phenylindole	LS	Leigh Syndrome
DNA	Deoxyribonucleic Acid	MAP2	Microtubule-Associated Protein 2
DNM1L	Dynamin 1-Like		

Abbreviations

mDANs	Midbrain dopaminergic neurons	NEUN	Neuron-Specific Nuclear Protein
MEA	Multi-Electrode Array	NESTIN	Neural Stem Cell Marker
MELAS	Mitochondrial Encephalomyopathy, Lactic Acidosis, and Stroke-like Episodes	NGN2	Neurogenin 2
MERRF	Myoclonic Epilepsy with Ragged Red Fibers	NR	Nicotinamide Riboside
MFN1	Mitofusin 1	NRF2	Nuclear Factor Erythroid 2-Related Factor 2
MFN2	Mitofusin 2	NSC	Neural Stem Cell
mHTT	Mutant Huntingtin	NPCs	Neural Progenitor Cells
MICOS	Mitochondrial Contact Site and Cristae Organizing System	NURR1	Nuclear Receptor-Related 1 Protein
MIRO1	Mitochondrial Rho GTPase 1	ODD	Orphan Drug Designation
MILS	Mitochondrial DNA-Related Leigh Syndrome	OCT3/4	Octamer-Binding Transcription Factor ¾
MMP	Matrix Metalloproteinase	OMM	Outer Mitochondrial Membrane
MO	Midbrain Organoid	OPA1	OPA1 Mitochondrial Dynamin-Like GTPase
MRI	Magnetic Resonance Imaging	OXPHOS	Oxidative Phosphorylation
MT-ATP6	Mitochondrial ATP Synthase Subunit 6	PDE5	Phosphodiesterase 5
mtDNA	Mitochondrial DNA	PD	Parkinson's Disease
mtUPR	Mitochondrial Unfolded Protein Response	PFO	Perfringolysin O
NAD ⁺	Nicotinamide Adenine Dinucleotide (oxidized form)	PGC-1α	Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1 Alpha
NADH	Nicotinamide Adenine Dinucleotide (reduced form)	PPAR	Peroxisome Proliferator-Activated Receptor
NAC	N-Acetylcysteine	PPARγ	Peroxisome Proliferator-Activated Receptor Gamma
NARP	Neurogenic Ataxia with Retinitis Pigmentosa	PPRE	Peroxisome Proliferator-Response Element
NDUFS1	NADH Dehydrogenase (ubiquinone) Fe-S Protein 1	Q	Glutamine
NDUFS4	NADH Dehydrogenase (ubiquinone) Fe-S Protein 4	RA	Retinoic Acid
nDNA	Nuclear DNA	RAR	Retinoic Acid Receptor
		RC	Respiratory Chain
		RNA	Ribonucleic Acid

ROS	Reactive Oxygen Species
RXR	Retinoid X Receptor
SCO2	Shunted Cytochrome Oxidase
scRNA-seq	Single-cell RNA sequencing
SHH	Sonic Hedgehog
SIRT1	Sirtuin 1
SNPH	Syntaphilin
SOX2	SRY-Box Transcription Factor 2
SURF1	Surfeit 1
SYBU	Syntabulin
TGF- β	Transforming Growth Factor Beta
TALEN	Transcription Activator-Like Effector Nuclease
TIM23	Translocase of the Inner Membrane 23
TFAM	Mitochondrial Transcription Factor A
TH	Tyrosine Hydroxylase
Wnt	Wingless-Int Pathway
WT	Wild-type
TRAK2	Trafficking Kinesin Binding Protein 2

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1. INTRODUCTION



1.1. MITOCHONDRIA AND THE BRAIN

Mitochondria are best known for being the powerhouse of eukaryotic cells, but their role extends far beyond energy production (Picard & Shirihi, 2022). This organelle is essential for maintaining calcium and reactive oxygen species homeostasis, regulating stemness, and controlling apoptosis (Picard & Shirihi, 2022). Given their involvement in these vital functions, it is not surprising that mitochondrial dysfunction is linked with a variety of human diseases (Suomalainen & Nunnari, 2024)

This is particularly evident for neurological disorders, where mitochondrial alterations have been reported in neurodevelopmental conditions such as autism spectrum disorder, Rett's and Fragile X syndromes (Khaliulin et al., 2025; Rojas-Charry et al., 2018; Valenti & Vacca, 2023). Mitochondrial dysfunction is also associated with psychiatric disorders, including depression (Jiang et al., 2024) and schizophrenia (Rajasekaran et al., 2015), as well as neurodegenerative diseases like Alzheimer's, Parkinson's, and Huntington's disease (Schon & Przedborski, 2011). Moreover, mutations affecting mitochondrial functions are causative of neurological disorders (DeBrosse & Parikh, 2012; Turnbull et al., 2010).

This highlights the crucial role of mitochondrial health in the proper development, function, and survival of the brain. Neurons, in particular, are highly vulnerable to mitochondrial dysfunction due to their reliance on mitochondrial oxidative phosphorylation to sustain membrane potential and synaptic function (Devine & Kittler, 2018).

Recognizing the fundamental role of mitochondria in neuronal development and function, research has increasingly focused on investigating the contribution of this organelle to brain health and disease, with growing interest in developing mitochondria-targeted therapies as a potential strategy for neurological disorders, resurfacing the so-called mitochondrial medicine (Picard et al., 2016).

1.1.1. Basic concepts of mitochondria

Mitochondria are double-membrane organelles composed of an outer mitochondrial membrane (OMM) and inner mitochondrial membrane (IMM), separated by an intermembrane space. The OMM is integral for trafficking proteins and the association of mitochondria with other organelles, such as the endoplasmic reticulum. The IMM, instead, plays a vital role in cellular energy production by housing a series of protein complexes within membrane invaginations called mitochondria cristae.

Mitochondrial cristae are folded structures of the IMM that extend into the mitochondrial matrix. Mitochondrial cristae house the mitochondrial respiratory chain complexes (RC) and enzymes that compose the electron transport chain (ETC) and are involved in adenosine triphosphate (ATP) synthesis. The highly folded architecture of mitochondrial

cristae maximizes energy output without increasing mitochondrial size, which is particularly important in energy-demanding cells like neurons and muscle fibers. Mitochondrial cristae allow the efficient organization of the molecular machinery required for energy production, ensuring that mitochondria function as the cell's primary powerhouse.

To obtain energy, nutrients such as glucose or lipids are metabolized in a series of cytosolic reactions where electrons are stripped off and fixed as Nicotinamide Adenine Dinucleotide (NADH) and Flavin Adenine Dinucleotide (FADH₂) coenzymes, which ultimately start an electron flow through the RC. The energy these reactions release is used by RC I, RC III and RC IV of the ETC to pump protons into the intermembrane space, generating an electrochemical gradient or mitochondrial membrane potential (MMP). This proton-derived energy is used by the mitochondrial ATP synthase (RC V) to phosphorylate ADP+Pi into ATP through a rotation-driven mechanism (Figure 1), a process known as oxidative phosphorylation (OXPHOS) (Alberts, 2002).

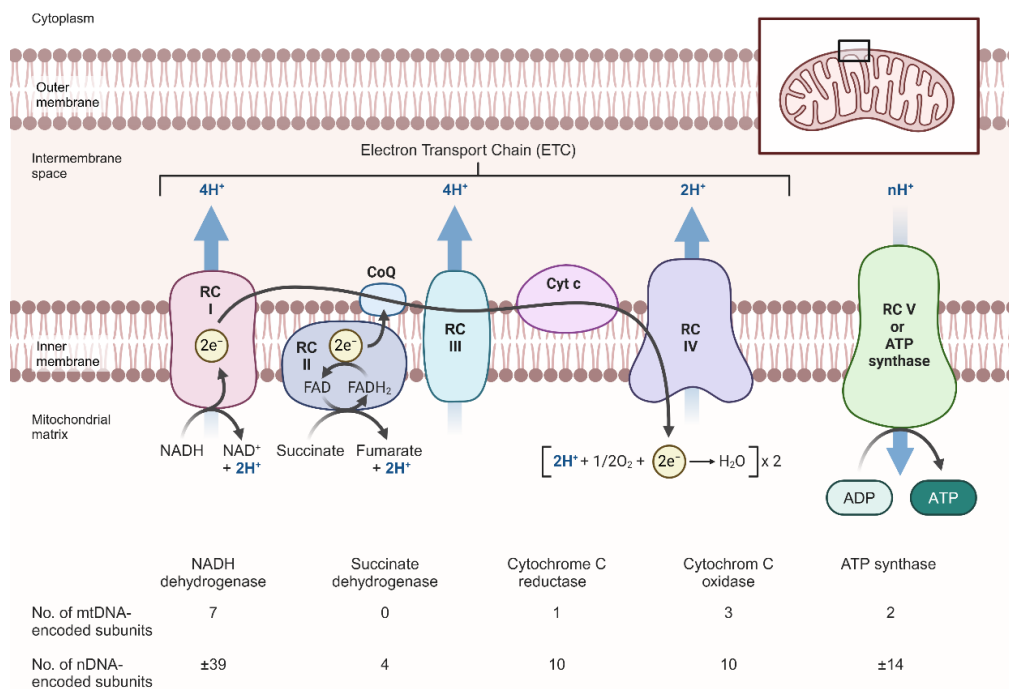


Figure 1. Schematic representation of the electron transport chain (ETC) and the respiratory complexes (RC) involved. The ETC is composed of RC I-IV, a series of protein complexes involved in the final stage of cellular energy production during aerobic respiration. In mitochondrial oxidative phosphorylation (OXPHOS), high-energy electrons (2e⁻) derived from previous metabolic reactions are shuttled along the RCs. The energy provided by the electron transfer is used to pump protons (H⁺) from the mitochondrial matrix into the intermembrane space, creating an electrochemical proton gradient, the mitochondrial membrane potential (MMP). This electrochemical gradient drives the ATP synthase to catalyze the synthesis of ATP. Numbers below show the number of subunits within each complex that are encoded by mitochondrial or nuclear DNA. This illustration was adapted from Biorender.

Each RC is formed by different protein subunits that require the orchestrated action of assembly factors to function properly (Vercellino & Sazanov, 2022). Mutations affecting either the generation of these structural proteins or their respective assembly factors can lead to dysfunctions in mitochondrial OXPHOS and defective energy production. These mutations can occur at the nuclear genome, where most structural and assembly proteins are encoded, or affect the small portion of DNA that remains in the mitochondria and that encodes for a small amount of ETC proteins (Vercellino & Sazanov, 2022).

The mitochondrial DNA (mtDNA), located in the mitochondrial matrix, is a double-stranded circular DNA comprising 16.5 kb in mammals that exists in multiple copies within the cell. Its full-length sequence was first determined by Anderson et al 1981 (Anderson et al., 1981), revealing that mtDNA encodes for 13 core subunits of the RC complexes (Figure 1), 22 tRNAs and 2 rRNA genes, which provide the RNA components for the translation of mtDNA-encoded proteins (Taanman, 1999). Hence, the vast majority of the mitochondrial proteome (around 1500 proteins) is encoded by nuclear genes, which are translated in the cytosol and further imported into the mitochondria (Calvo & Mootha, 2010).

The existence of these two genomes demands a tight cross-talk between the nuclear DNA (nDNA) and the mtDNA for the generation of new mitochondria (Scarpulla, 2008), a process called mitochondrial biogenesis (Figure 2). Mitochondrial biogenesis is a complex process that involves mtDNA transcription and translation, the import of nuclear-encoded proteins into the mitochondria, and inter-organellar transport of newly synthesized lipids (Pfanner et al., 2019), with the ultimate goal of ensuring an optimal mitochondrial mass in the cell.

Maintaining an optimal mitochondrial mass is crucial to sustaining energy production and overall cellular homeostasis. For this, the mitochondrial content within the cell is tightly regulated by equilibrating the synthesis and removal of this organelle in a process called mitophagy (Figure 2). Mitophagy is a vital quality control mechanism to avoid cell death, which can occur upon severe mitochondrial damage, such as in cases of pathological situations leading to oxidative stress (Ashrafi & Schwarz, 2013).

Mitochondrial OXPHOS is a major source of reactive oxygen species (ROS), highly reactive molecules capable of damaging proteins, nucleic acids, and lipids. Under physiological conditions, ROS act as signaling molecules involved in cell proliferation, development, immunity, apoptosis, among others (Antonucci et al., 2021). However, excessive ROS production can lead to oxidative stress, causing irreversible cellular damage, including lipid peroxidation, DNA modifications, and mitochondrial energy production failure, which are commonly observed in brain disorders (Angelova & Abramov, 2018).

ROS levels are tightly regulated by intracellular antioxidant enzymes, including glutathione peroxidase, superoxide dismutase or peroxiredoxin (Antonucci et al., 2021). Mutations affecting the ETC can lead to excessive ROS production that overwhelms these intracellular pathways (Hayashi & Cortopassi, 2015; Lake et al., 2015).

Upon oxidative stress, accumulation of misfolded or damaged proteins in the mitochondria can trigger an additional quality control mechanism: the mitochondrial unfolded protein response (UPR_{mt}). The UPR_{mt} activates a transcriptional program that enhances mitochondrial proteostasis by increasing chaperone and protease expression, ultimately preserving mitochondrial function under stress conditions (Shadel & Horvath, 2015).

In addition to mitochondrial biogenesis and mitophagy, the quality and quantity of mitochondria can be modulated through mitochondrial dynamics (Figure 2). Mitochondria are highly dynamic organelles that continuously undergo processes of fission and fusion to maintain the mitochondrial network and respond to stimuli (Chan, 2006). For example, during conditions of high energy demand, the fusion of mitochondria allows the exchange of gene products and metabolites that enhance their overall respiratory function (Liesa & Shirihai, 2013). Instead, mitochondrial fission allows the release of damaged components for autophagy or the release of cytochrome C during apoptosis.

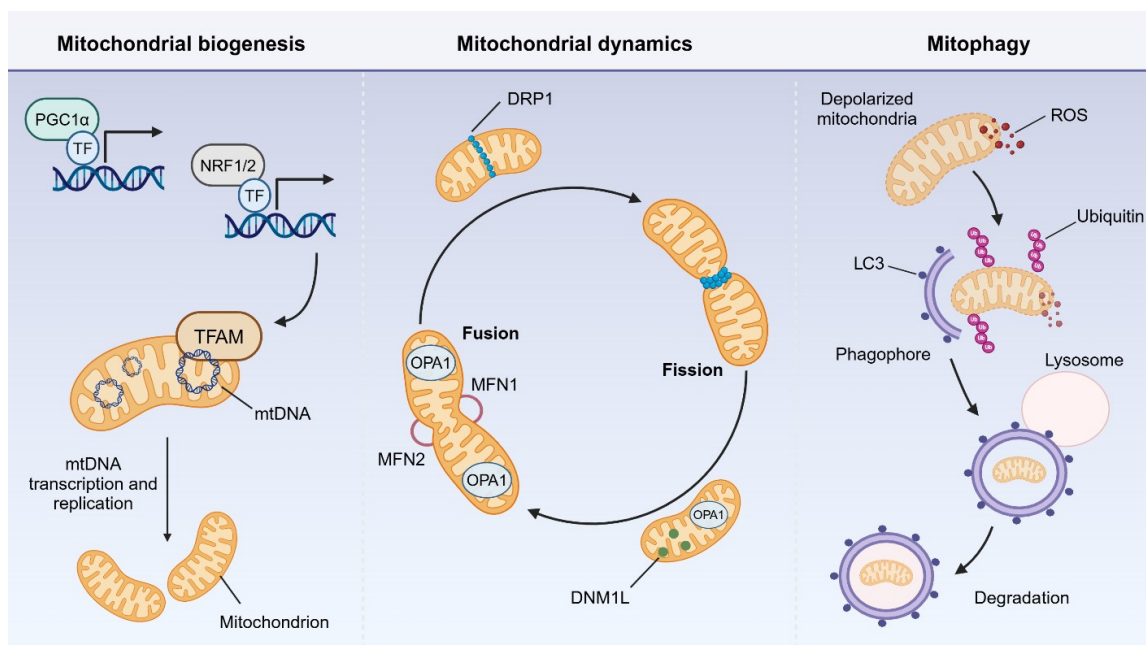


Figure 2. Mitochondrial quality control mechanisms. Upregulation of the master regulator of mitochondrial biogenesis PGC-1 α and associated transcription factors (TFs) leads to the transcription of nuclear-encoded mitochondrial proteins. Among these, TFAM is imported into the mitochondria to upregulate the expression of mitochondrial-encoded ETC subunits, resulting in increased oxygen consumption, ATP synthesis, and mitochondrial content. Changes in mitochondrial morphology are under the control of fusion (MFN1, MFN2, OPA1) and fission (DNM1L) proteins. These factors upregulate mitochondrial turnover by facilitating the dilution and clearance of damaged organelles. Mitochondrial components are eventually recycled through a specialized autophagic pathway known as mitophagy. DNM1L, dynamin 1-like protein; LC3, microtubule-associated proteins 1A/1B light chain 3; MFN1, mitofusin 1; MFN2, mitofusin 2; NRF1, nuclear respiratory factor 1; NRF2, nuclear factor erythroid 2-related factor 2; OPA1, mitochondrial dynamin-like 120 kDa protein; PGC-1 α , proliferator-activated receptor- γ co-activator 1 α ; ROS, reactive oxygen species; TFAM: mitochondrial transcription factor A. Illustration created with Biorender, adapted from (Picca et al., 2018)

Importantly, these dynamic processes are accompanied by remodeling of the mitochondrial cristae. This remodeling process is regulated by key structural and functional components, such as mitochondrial contact site and cristae organizing system (MICOS), OPA1, the F1Fo ATP synthase, and the local lipid microenvironment. Changes in cristae architecture and local fluctuations in MMP at the level of individual cristae are predicted to have major consequences for mitochondrial functions such as OXPHOS, calcium homeostasis, and apoptosis (Kondadi et al., 2020).

Maintaining a balance of these processes is essential for sustaining the multifaceted nature of mitochondria in cellular homeostasis. Overall, a healthy and efficient mitochondrion requires nuclear-mitochondrial cross-talk to obtain numerous molecular components (e.g. proteins, lipids) that can correctly interact with one another to perform specific enzymatic activities (e.g. ETC activity) or generate intrinsic properties (e.g. MMP) that combined finally result on several mitochondrial functions (e.g. OXPHOS, calcium homeostasis) (Monzel et al., 2023).

These mitochondrial functions have evolved to adapt to the specific needs of different cell types, by diversifying their composition, organization and physiology, and giving rise to the existence of distinct intracellular and intercellular mitotypes (Menacho & Prigione, 2020; Suomalainen & Nunnari, 2024). Hence, it is becoming increasingly relevant to investigate their specialized roles and characteristics in distinct cell types to understand how these contribute to specific cellular vulnerabilities upon mitochondrial dysfunction.

1.1.2. Neuronal mitochondria

The human brain consumes approximately 20% of the total energy expenditure of the body although representing only 2% of the body weight, with even higher metabolic requirements during development (Goyal et al., 2014; Kuzawa et al., 2014). The brain's high energy demand is primarily needed to support neuronal activity, namely the generation of action potentials, the repolarization of the membrane potential, and the maintenance of synaptic activity (Attwell & Laughlin, 2001).

To meet such great energy requirements, neurons depend on a continuous influx of substrates from blood to locally and instantly generate ATP. To fuel these functions, neurons use glucose as their primary energy source, although alternative fuels such as ketone bodies and lactate can be utilized in specific circumstances, such as intense physical activity, fasting, or during developmental stages (Camandola & Mattson, 2017).

In neurons, glucose is metabolized via glycolysis and the pentose phosphate pathway, a necessary balance important for sustaining the availability of nicotinamide adenine dinucleotide phosphate (NADPH) and safeguarding neurons from oxidative stress and cell death (Herrero-Mendez et al., 2009). The glycolytic process metabolizes glucose to pyruvate, which can be actively transported into the mitochondria to be converted to acetyl

coenzyme A (acetyl-CoA), which is further processed via the Krebs cycle and ultimately fueling OXPHOS (Camandola & Mattson, 2017).

Neurons are therefore predominantly oxidative and heavily rely on mitochondrial OXPHOS for energy production. Given that ATP has a limited diffusion capacity, mitochondria need to be actively transported and docked in specific subcellular locations to support the high energetic neuronal needs (Devine & Kittler, 2018).

In this regard, neurons are highly polarized cells with unique structures, such as the axon and dendrites, designed to process and transmit electrochemical signals. Dendrites emanate from the cell body (neuronal soma) and bifurcate repeatedly to recreate complex tree-like structures, while the axon is typically a long, slender projection that transmits action potentials away from the soma towards the axon terminal, where presynaptic buttons loaded with neurotransmitters are released to communicate with surrounding neurons (Richards et al., 2018)

Importantly, axons and dendrites are distinguished not only by their morphology and function but also by the expression of specific molecular markers. For instance, Tau protein is enriched in axons, whereas MAP2 is predominantly localized to dendrites, allowing for their molecular identification during development and in experimental studies (Kirkpatrick LL & Brady ST, 1999).

Mature neurons have highly diverse and complex dendritic arborization, displaying differences in neurite length, the degree of neurite branching, and its spatial distribution. These structures determine how current flows into and through the cell and, therefore, modulate how information is received, processed, encoded, and further transmitted to other cells (Nicholls et al., 2012). Thus, proper development and maintenance of neuronal morphology and arborization are crucial for the correct functioning of neuronal synaptic transmission (Richards et al., 2018). Structural changes in neuronal cytoarchitecture can profoundly impact neural circuit formation, synaptic connectivity, and brain function, as commonly observed in neurodevelopmental and psychiatric disorders (Forrest, Parnell & Penzes, 2018).

Neurons with highly ramified dendritic structures and long axons can cover vast areas, playing a central role in communication over long distances and contacting thousands of synapses, requiring a distinctive spatiotemporal energy distribution. In this context, mitochondrial transport and docking are vital for the development and maintenance of neuronal morphology and arborization (Sheng & Cai, 2012), as well as regulating synaptic activity (Rangaraju et al., 2014; Vaccaro et al., 2017) (Figure 3).

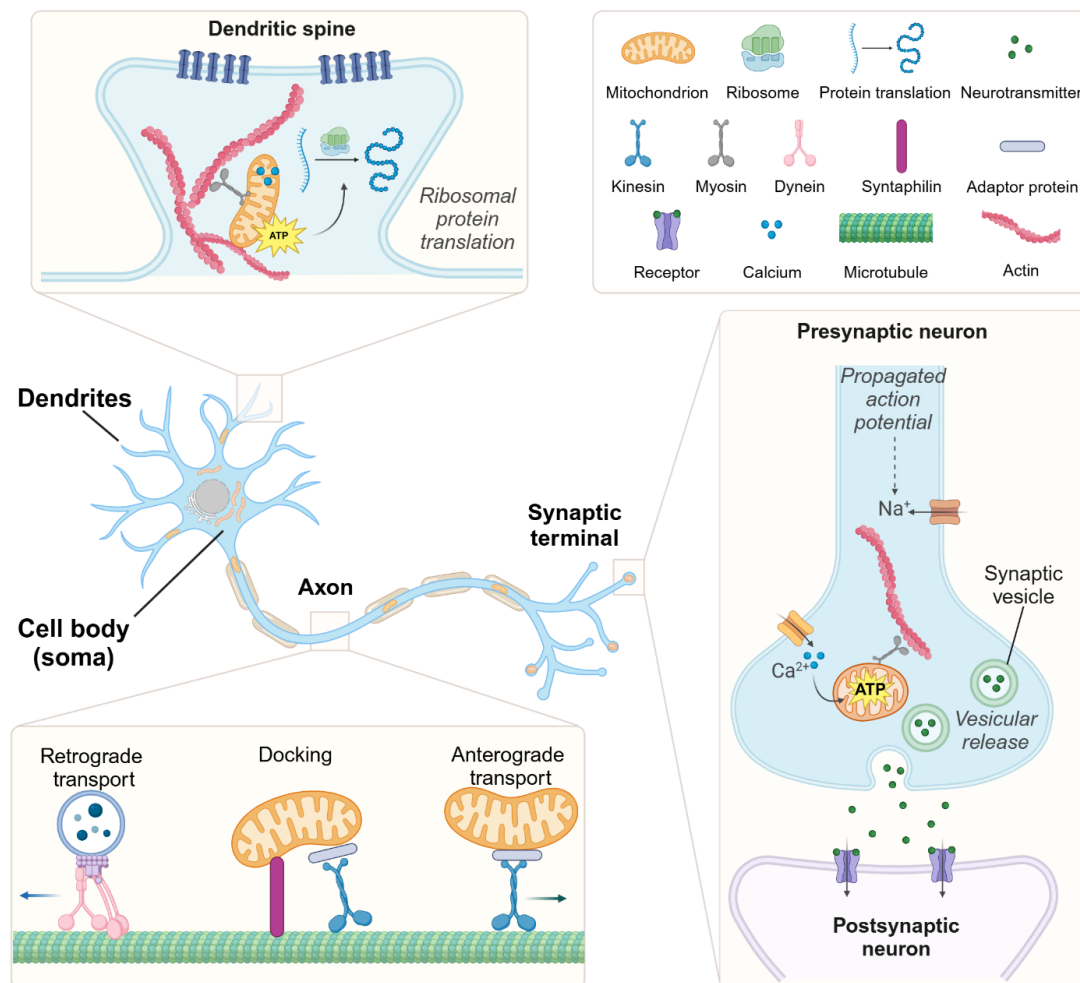


Figure 3. Mitochondrial transport ensures proper spatiotemporal energy distribution that fuels neuronal branching and synaptic activity. Neurons have three distinct functional and structural domains: the soma, the axon, and the ramified dendritic arbors. Through specialized cellular mechanisms, mitochondria can be transported and remain stationary in these strategic domains to sustain their high energy demand and calcium homeostatic plasticity. Long-range mitochondrial transport from the soma to distal axonal and dendritic regions depends on neuronal microtubules. In axons, dynein motors are responsible for returning mitochondria to the soma, while kinesin motors of the KIF5 family drive anterograde mitochondrial transport to distal axonal regions and synaptic terminals, and syntaphilin (SNPH) mediates the recruitment of this organelle to stationary pools. Mitochondria docking ensures that mitochondria are adequately distributed within axons and synapses, allowing neurite branching and energy production on-site. In presynaptic terminals, small, punctate mitochondria fuel synaptic activity and control calcium homeostasis. Instead, long and tubular mitochondria at dendritic spines regulate calcium levels via mitochondria-endoplasmic reticulum contacts (not shown) to fuel local protein translation, aiding synaptic plasticity. Illustration created with Biorender, adapted from (Sheng & Cai, 2012).

To reach these specific subcellular locations, distinct molecular mechanisms governing mitochondrial motility and anchoring are described in axons and dendrites (Figure 3). For example, Syntaphilin (SNPH) has been described as an axonal-specific mitochondrial anchor protein (Kang et al., 2008). The reduction or deletion of SNPH increases mitochondrial motility in axons, decreasing axonal branching (Courchet et al., 2013), and impairing mitochondria positioning at synapses, affecting synaptic function (Kang et al.,

2008). Instead, overexpression of SNPH leads to increased mitochondrial anchoring with enhanced axonal branching (Courchet et al., 2013). However, the aberrant expression of SNPH in dendrites causes neurodegeneration (Joshi et al., 2019), revealing this differential regulatory mechanism. Similarly, the mitochondrial transport protein Miro1 (Mitochondrial Rho GTPase 1) is crucial for regulating the development and maintenance of the dendritic arbor in neurons but is not involved in the mitochondrial distribution along the axon (López-Doménech et al., 2016).

Although the molecular mechanisms behind the localized recruitment of mitochondria are still being elucidated, mitochondrial calcium signaling plays a pivotal role in regulating mitochondrial positioning in synapses via energy-sensing molecular mechanisms (Li et al., 2020). Functioning as high-capacity calcium buffers, mitochondria regulate calcium homeostasis in neurons (Figure 3). During synaptic transmission, the depolarization of the neuronal membrane causes an influx of calcium that allows the fusion of synaptic vesicles and the release of neurotransmitters. At the same time, calcium levels in synaptic mitochondria increase, leading to enhanced mitochondrial ATP production to fuel synaptic vesicle recycling (Rangaraju et al., 2014). This, in turn, buffers cytosolic calcium, and regulates synaptic activity, preventing excitotoxicity (Vaccaro et al., 2017).

Altogether, the strategic location and morphological adaptability of mitochondria are essential to meet their function as key regulators of calcium homeostasis and energy supply in neurons (Rangaraju et al., 2019).

Beyond their role in neuronal morphogenesis and function, mitochondrial dynamics and metabolism are emerging as key regulators of neurogenesis (Iwata & Vanderhaeghen, 2021). Neurogenesis is the process in neural development by which stem cells stop self-renewing and dividing to commit, exit the cell cycle, and become postmitotic neurons. Many intrinsic and extrinsic cues, such as transcription factors and morphogens, are known drivers of neurogenesis (Bonnetfont & Vanderhaeghen, 2021; Villalba et al., 2021), but recent studies highlight the role of mitochondria in this event.

In the developing mouse cortex, mitochondrial fission varies across neurogenic stages: stem cells exhibit mostly fused mitochondria, more committed progenitor cells show fragmented mitochondria, and newborn neurons display even smaller mitochondria (Figure 4) (Iwata et al., 2020; Khacho et al., 2016).

Genetic manipulations that promote mitochondria fusion and fission proved that these morphological changes actively regulate the neuronal differentiation. In this context, disruption of well-known proteins involved in mitochondrial fusion, such as OPA1 (optic atrophy 1) and MFN1/2 (mitofusin 1 and 2), led to decreased stem cell self-renewal and increased neuronal differentiation; whereas disruption of mitochondrial fission via manipulation of DRP1 (dynamin-related protein1) led to increased self-renewal and decreased neurogenesis (Iwata et al., 2020; Khacho et al., 2016).

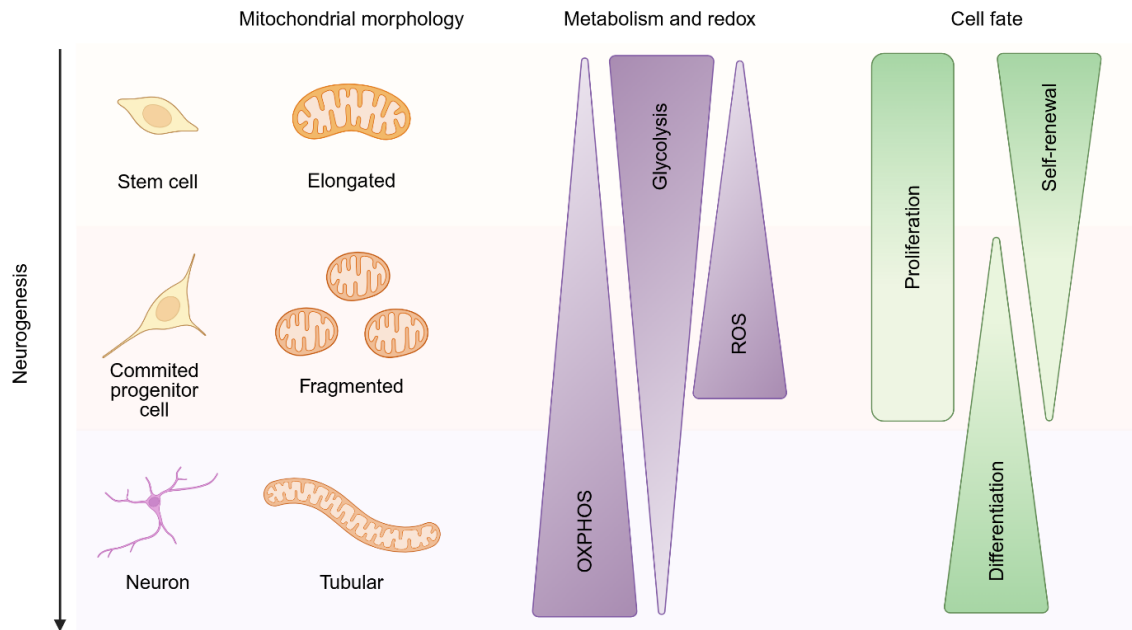


Figure 4. Schematic representation of changes in mitochondrial structure and function during embryonic neurogenesis. During neurogenesis, cells progress from stem cells towards more committed progenitor cells and onto neurons, becoming progressively more dependent on OXPHOS while relying less on glycolysis. The transition from stem cell to committed progenitors also involves the production of ROS, which are involved in proliferation, self-renewal and cell survival signaling. Additionally, mitochondrial morphology undergoes dynamic changes during differentiation, becoming more fragmented during the NPC phase and then elongated again during differentiation into neurons. Illustration created with Biorender, adapted from (Khacho et al., 2019).

Mitochondrial dynamics are tightly linked to metabolism via the regulation of mitochondrial cristae, where ETC complexes are located. Differentiating cells exhibit distinct metabolic profiles (Figure 4): stem cells predominantly rely on aerobic glycolysis for their fast kinetics, whereas mature neurons heavily depend on OXPHOS for energy production as the most efficient way to generate ATP (Iwata & Vanderhaeghen, 2021). Thus, neurogenesis requires a metabolic shift towards OXPHOS, coupled with mitochondrial morphological remodeling, to support neuronal morphogenesis and functionality (Brunetti et al., 2021; Iwata & Vanderhaeghen, 2021).

The findings above-mentioned pose mitochondria as keepers of neuronal synaptic function and plasticity in the adult brain, while revealing their emerging role in neuronal commitment and development (involved in neurogenesis, neuritogenesis, and synaptogenesis). Understanding the mechanisms linking mitochondrial dysfunction to early neural defects is therefore crucial. In this thesis, these mechanisms will be explored in the context of Huntington's disease (HD) and Leigh syndrome (LS)

1.2. HUNTINGTON'S DISEASE

Huntington's disease (HD) (OMIM #143100) is a genetic neurodegenerative disorder characterized by a combination of motor, psychiatric, and cognitive symptoms (Walker, 2007). Clinically, HD presents a range of involuntary movements, including chorea, dystonia, and bradykinesia, alongside progressive cognitive decline ultimately leading to dementia (Walker, 2007).

The disease primarily affects the basal ganglia, a group of subcortical nuclei located beneath the outer cortical layer of the cerebral cortex that controls movement (Figure 5A). The basal ganglia regulate posture, counteract tremors, and maintain steady muscular contractions throughout a highly interconnected and complex functional circuitry involving glutamatergic, GABAergic and dopaminergic neurons (Figure 5B).

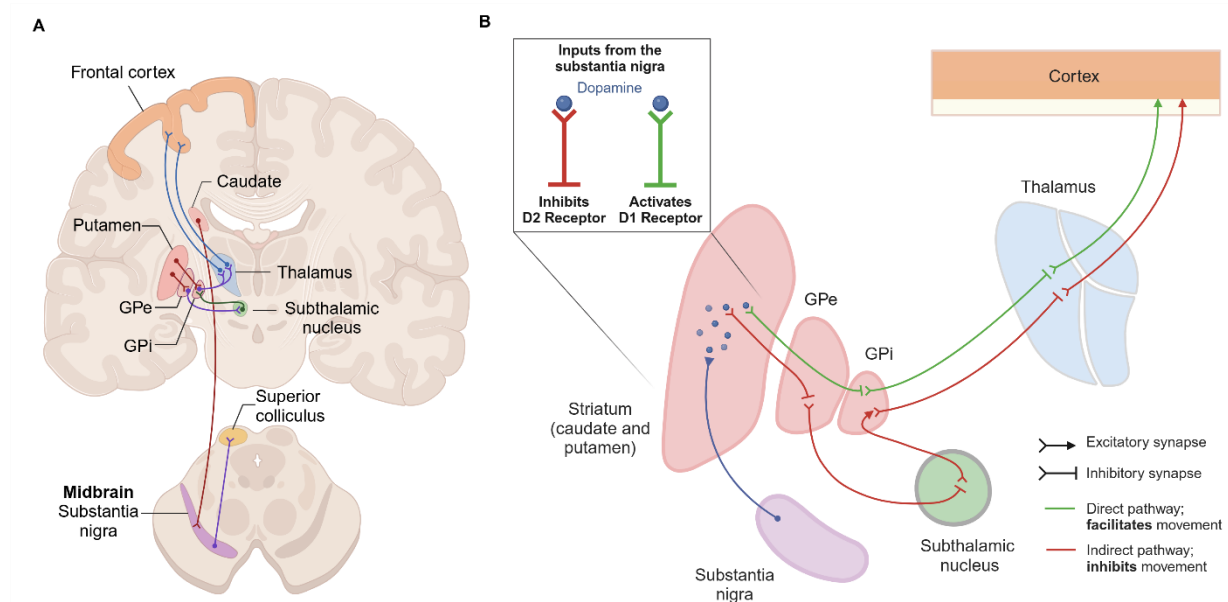


Figure 5. The basal ganglia circuitry. **A** The main functional components of the basal ganglia include the striatum (formed by the caudate nucleus and putamen), the globus pallidus (external and internal (GPe and GPI, respectively) divisions), the subthalamic nucleus and the substantia nigra (located in the midbrain). **B** Dopaminergic neurons of the substantia nigra can have a differential impact on medium spiny neurons of the striatum, depending on the expression of dopaminergic receptors (D1 or D2). D1-expressing medium spiny neurons are involved in the *direct pathway*, and they inhibit GABAergic neurons of the internal globus pallidus, resulting in a net excitation of the thalamus that projects to the cortex and facilitates motor actions. D2-expressing medium spiny neurons involved in the *indirect pathway*, by which they inhibit GABAergic neurons of the external globus pallidus, indirectly inhibiting the thalamus and therefore resulting in global motor inhibition and termination of responses (Nicholls et al., 2012). In HD, medium spiny neurons of the striatum are particularly vulnerable to dysfunction and neurodegeneration, firstly affecting those involved in the ‘indirect pathway’ early in the course of the disease, while affecting those involved in the ‘direct pathway’ at later stages. Remarkable, most interneurons located in the striatum remain largely spared, eliciting a special vulnerability of certain neuronal subtypes (Han et al., 2010). Illustration created with Biorender.

Structurally, the basal ganglia sit below the cortex and above the midbrain region, which holds important structures such as the substantia. The midbrain and the basal ganglia are strongly connected through afferent and efferent connections that form a complex feedback circuitry that regulates movement (Nicholls et al., 2012).

In HD, neurodegeneration primarily affects the dorsal striatum and the substantia nigra, eventually extending to cortical regions (Reiner et al., 1988). The observed neurodegeneration is accompanied by neuroinflammation, characterized by the activation of astrocytes and microglia (Ghosh & Tabrizi, 2018).

The genetic cause of HD is the expansion of CAG repeats in exon 1 of the huntingtin (HTT) gene, located on chromosome 4. The CAG repeats encode for the N-terminal polyglutamine segment of the HTT protein that contains 6-35 glutamine (Q) residues in its wild-type form. The pathogenic form of HTT (also known as mutant HTT, mHTT) is defined when a threshold of >40Q is exceeded. The onset and clinical severity of the disease are proportional to the number of CAG repeats, with longer repeats leading to more severe symptoms occurring earlier in life (The Huntington's Disease Collaborative Research group, 1993).

The prevailing hypothesis in the field is that excessive elongation of the polyQ tail causes mHTT protein aggregation, leading to aberrant interaction with other proteins and the disruption of multiple cellular functions, conferring it a toxic gain-of-function (Jones & Hughes, 2011).

An alternative or complementary hypothesis posits that HD pathogenesis also involves a loss of function of wild-type HTT (Cattaneo et al., 2005). Human wild-type HTT is a ubiquitous protein, highly expressed in the brain (Sharp et al., 1995). In neurons, HTT is essential for development and survival (Reiner et al., 2003) and has been implicated in mitochondrial and vesicular transport (Gauthier et al., 2004; Trushina et al., 2004), synaptic function (Buren et al., 2016; Usdin et al., 1999), and gene regulation (Kegel et al., 2002). HTT portrays an extensive interactome, comprising over 200 known proteins (Li & Li, 2004), and its full functional overview remains incomplete. Thus, the lack of a functional HTT protein may impair cellular physiological, leading to cellular dysfunction and neuronal degeneration.

This dual mechanism—gain of toxic function and loss of physiological function—likely contributes to the complex molecular pathology of HD. However, the precise interplay between these processes and the mechanism by which they lead to selective vulnerability and neurodegeneration of striatal medium spiny neurons and dopaminergic neurons, while sparing other neuronal subtypes, remains a subject of ongoing investigation.

1.2.1. Mitochondrial dysfunction

Mitochondrial dysfunction is a hallmark of HD pathology (Carmo et al., 2018). HTT has been described to be involved in mitochondrial function and early studies identified mitochondrial alterations as a hallmark of HD, linking mitochondrial pathology to striatal neurodegeneration and promoting extensive research into its role as driver of disease pathology (Bossy-Wetzel et al., 2008; Jurcau & Jurcau, 2023).

In 1978, the first study describing ultra-structural abnormalities in mitochondrial morphology in a post-mortem brain sample of an individual suffering from HD was released (Goebel et al., 1978). A few years later, one of the first animal models of HD was achieved by administering the RC II reversible inhibitor 3-NP, which caused HD-like locomotor symptoms and striatal cytotoxicity (Beal et al., 1993; Brouillet et al., 1995; Kodsi & Swerdlow, 1997).

Since then, extensive research has documented mitochondrial alterations in various models, ranging from human samples to animal studies, at different stages of the disease – both preclinical and clinical (Carmo et al., 2018). These studies report defects in mitochondrial metabolism, mitochondrial calcium handling, mitochondrial biogenesis, dynamics, mitophagy, and apoptosis, highlighting the widespread nature of mitochondrial dysfunction throughout the disease (Figure 6).

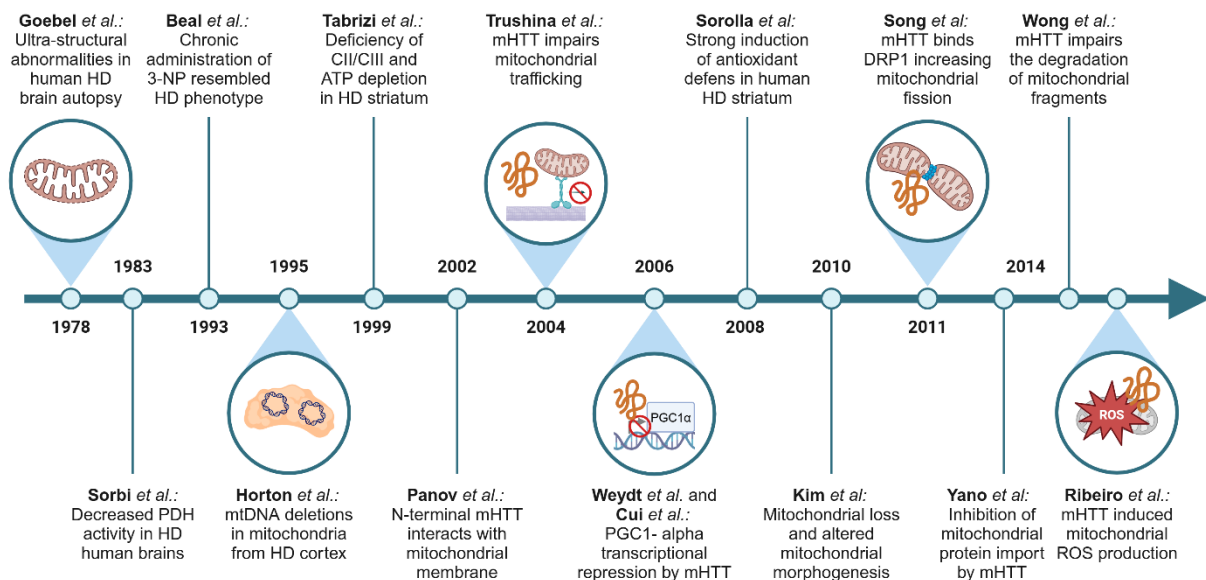


Figure 6. Timeline with key discoveries involving mitochondria in HD pathogenesis. Major discoveries depicting mitochondrial dysfunction in HD, from dysfunctional glycolysis and deficiency of respiratory chain complexes in HD patients, to later discoveries describing direct interactions between mHTT and mitochondria. All studies are indicated in the reference list. Illustration created with Biorender, adapted from (Carmo et al., 2018).

Notably, latest research has been able to report direct links between mHTT and mitochondrial dysfunction, thereby placing mitochondrial dysfunction as a key process in the neuropathological cascade in HD (Bossy-Wetzel et al., 2008; Jurcau & Jurcau, 2023).

Importantly, targeting mitochondrial alterations has been shown to stop disease progression and have neuroprotective effects. A pivotal study by Yano et al. revealed that mHTT inhibits mitochondrial protein import via the interaction with TIM23, a mitochondrial protein import complex (Yano et al., 2014). This alteration occurred even in a presymptomatic mouse model of HD, and restoring TIM23-mediated protein import into the mitochondria mitigated neuronal death (Yano et al., 2014).

This study suggests that mitochondrial dysfunction is not a mere consequence of the disease but rather a critical upstream element in the cascade of events leading to neuronal pathology, emphasizing the therapeutic potential of mitochondrial-based therapies for HD.

In addition to the toxic gain-of-function mechanism, the loss of physiological functions of HTT could lead to mitochondrial dysfunction. Wild-type HTT has been implicated in multiple cellular functions, including vesicle trafficking, autophagy regulation, and mitochondrial dynamics (Cattaneo et al., 2005; Ross & Tabrizi, 2011). Accordingly, loss of these physiological functions can lead to defective mitochondrial trafficking and distribution within neurons, increased mitochondrial fission, reduced ATP production, and increased oxidative stress, ultimately impairing neuronal survival (Costa et al., 2010; Knott et al., 2008; Reddy & Shirendeb, 2012; Song et al., 2011).

Additionally, HTT has been linked to transcriptional regulation of genes involved in mitochondrial biogenesis and function, suggesting that mHTT-mediated transcriptional dysregulation may contribute to the energy deficits observed in HD (Cui et al., 2006; Gines et al., 2003).

Current perspectives on neurodegenerative diseases suggest that neuronal dysfunction begins early in disease progression, even in asymptomatic individuals. Over time, the accumulation of cellular damage leads to selective neurodegeneration and the manifestation of the known symptomatology (Walker, 2007). Developing early pharmacological strategies to treat the disease in the prodromal phase, before extensive neuronal loss occurs, would be highly effective (Tabrizi et al., 2022).

To achieve this, it is essential to elucidate the pathophysiological mechanisms by which mutations in HTT lead to early neuronal dysfunction (Makeeva et al., 2023). Moreover, given the newly found roles of mitochondria in driving neuronal development and the link between mHTT and mitochondria, the impact of mHTT in neuronal development needs to be assessed to understand how this can contribute to early neuronal dysfunction seen in HD patients.

1.3. MITOCHONDRIAL DISEASES: LEIGH SYNDROME

Mitochondrial diseases encompass a diverse group of progressive, genetically transmitted, multisystemic disorders caused by impaired mitochondrial function (Gorman et al., 2016). These disorders can manifest at any age and affect virtually any organ, particularly those highly dependent on aerobic metabolism, such as the brain, muscles, and heart (Gorman et al., 2016; McFarland et al., 2010). Current treatments for mitochondrial diseases focus on symptomatic management and no disease-modifying therapies exist for the vast majority of mitochondrial disorders (Kornblum et al., 2023).

Mutations in either nDNA or mtDNA cause mitochondrial disorders. Nuclear mutations may affect a wide range of proteins involved in mitochondrial functions, such as assembly factors of RC complexes, proteins involved in mtDNA replication, maintenance, and transcription, as well as proteins responsible for mitochondrial dynamics, mitophagy, apoptosis, and OXPHOS homeostasis (Koopman et al., 2013). mtDNA mutations, instead, directly affect RC complexes. These mutations can be homoplasmic, when all copies of mtDNA carry the mutation, or heteroplasmic, when mutant mtDNA coexists with wild-type mtDNA (DeBrosse & Parikh, 2012).

While homoplasmic mutations lead to disease in all cases, the clinical presentation of heteroplasmic mutations may vary depending on the mutation load (DeBrosse & Parikh, 2012). For instance, patients with the m.8993T>G mutation in the *MT-ATP6* gene may present mild symptoms and be diagnosed with NARP syndrome (Neurogenic weakness, Ataxia, and Retinitis Pigmentosa) when the level of heteroplasmy is lower than 80%, and usually manifest more severe symptoms and be diagnosed with LS when the level of mutation exceeds 80-90% of heteroplasmy (Rahman, 2023).

Therefore, in addition to the extensive genetic heterogeneity, the clinical presentation of mitochondrial diseases can be highly heterogeneous, displaying tissue-specific manifestations even when mutations affect the same mitochondrial complex, showing limited genotype-phenotype correlation with a wide range of clinical manifestations (Gorman et al., 2016; Lake et al., 2016).

The most common manifestation of a primary mitochondrial disorder is Leigh syndrome (LS), first described as subacute necrotizing encephalomyelopathy by David Leigh (Leigh, 1951).

LS (OMIM #256000) is a rare, inherited neurological disorder that affects approximately 1 in 40,000 live births (Rahman et al., 1996). The disease typically presents in infants who may suddenly face a situation of metabolic decompensation (such as an infection or illness) that prompts the appearance of symptoms, often during the first year of their life. Initial symptomatology includes poor feeding, vomiting, and failure to thrive, followed by more pronounced neurological deficits such as developmental delay, hypotonia, ataxia, and

dystonia. As the disease progresses, patients may experience further neurological deterioration, including optic atrophy, nystagmus, and epilepsy (Rahman, 2023).

While LS predominantly affects the CNS, some patients may also display extra-neurological symptoms, including cardiac, hepatic, or renal involvement, depending on the specific genetic mutation and clinical course (Mancuso, 2023). Given the complex interplay between genetic factors, tissue-specific expression, and environmental triggers, the severity and progression of symptoms can vary widely, even among individuals with the same genetic mutation (Lake et al., 2016).

LS has been reported to be caused by defects of 16 mitochondrial genes and approaching 100 nuclear genes (Rahman, 2023). Among these, mutations in the nuclear gene *SURF1*, involved in the assembly and stabilization of RC IV, are one of the most frequently identified causes of the disease (Rahman, 2023; Stenton et al., 2022).

1.3.1. *SURF1* gene mutations

The *SURF1* gene, located on the long arm of chromosome 9 (Zeviani et al., 1999), encodes a highly conserved 300 amino acids protein involved in the early stages of assembly of RC IV (also known as cytochrome C oxidase, COX) of the mitochondrial ETC. In the ETC, RC IV catalyzes the oxidation of cytochrome C while simultaneously reducing oxygen to water, using the energy released by the reaction to pump protons across the mitochondrial membrane. Therefore, as the terminal enzyme of the ETC, RC IV acts as a rate-limiting enzyme that controls the MMP and the overall mitochondrial respiration (Dalmonte et al., 2009; Villani et al., 1998).

Mammalian RC IV (from bovine heart) consists of 14 subunits, with 11 encoded by nDNA and 3 of them (MT-COX1, MT-COX2 and MT-COX3) by mtDNA (Soto et al., 2012). Although the RC IV biogenesis process is not entirely clear, it is known that MT-COX1 and MT-COX2 constitute the hydrophobic catalytic core of the enzyme, whereas the remaining subunits stabilize the catalytic core and its activity (Wikström & Sharma, 2018). The assembly of RC IV requires more than 30 known assembly factors, which are conserved from yeast to humans, underscoring the evolutionary conservation of the process (Barrientos et al., 2009; Williams et al., 2004). However, the exact function of *SURF1* in RC IV assembly process remains poorly understood (Soto et al., 2012).

Over 80 mutations in the *SURF1* gene have been documented, and nearly 40% of these are pathogenic (Lee & Chiang, 2021; Ruhoy & Saneto, 2014) (Figure 7). These mutations – nonsense mutations, aberrant splicing, or frameshift deletions – can lead to mRNA instability or the production of truncated forms of the *SURF1* protein that are rapidly degraded, ultimately leading to impaired assembly of RC IV (Kovářová et al., 2012; Pecina et al., 2003).

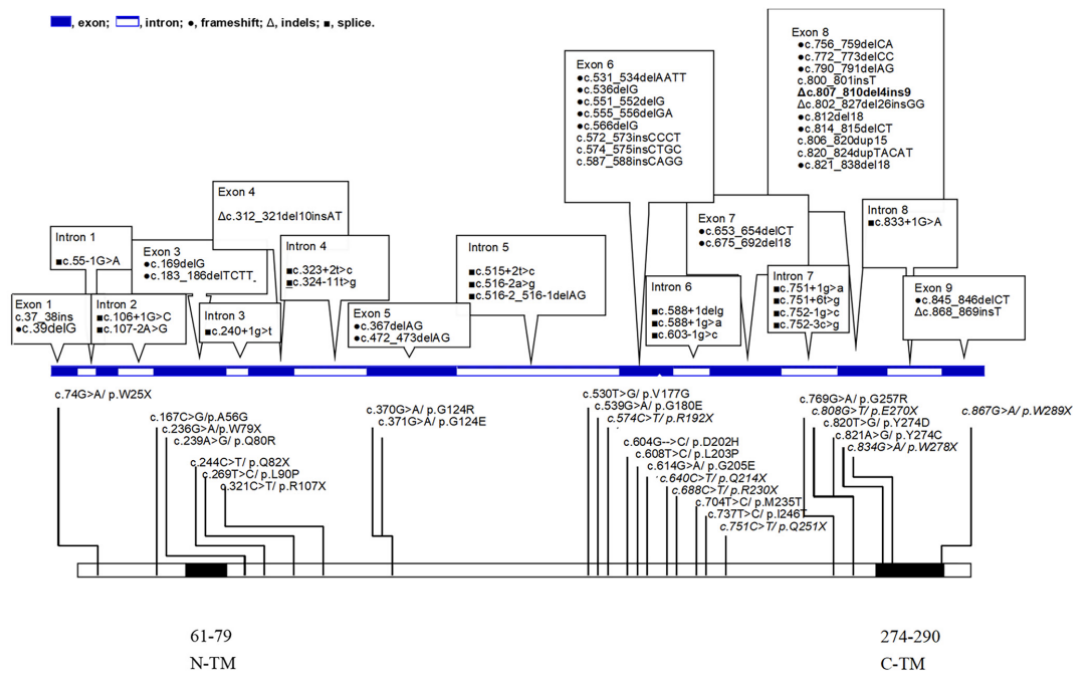


Figure 7. Distribution of *SURF1* mutations based on published literature. Reproduced from (Lee & Chiang, 2021).

An impaired assembly of RC IV would affect the overall energy production and alter ROS homeostasis. Being the terminal enzyme of the ETC and regulating the MMP, RC IV deficiency would have far-reaching implications given the role of MMP on import of nuclear-encoded proteins, synthesis of iron-sulfur clusters, the regulation of mitochondrial dynamics, and maintenance of cristae integrity (Youle & Van Der Bliek, 2012; Zorova et al., 2018). Additionally, these molecular defects would activate distinct compensatory mechanisms in the cell to reestablish balance and meet the energetic needs, such as changes in protein expression or a shift towards alternative metabolic pathways.

RC IV activity and the expression of the different subunits and cofactors are dynamic, varying between tissues with different metabolic demands (Kovářová et al., 2012; Sinkler et al., 2017). This tissue-specific regulation of RC IV may partially explain the differential impact of mutations in various genes. For example, mutations in *SCO1* tend to primarily affect the liver, while mutations in *SCO2* or *COX15* more commonly affect the heart (Böhm et al., 2006). Mutations in *SURF1*, however, are most often associated with neurological impairment, particularly affecting the brain (Böhm et al., 2006; Ruhoy & Saneto, 2014).

Additionally, the extent of the functional impairment may vary depending on the degree of RC IV subunit assembly disruption and the compensatory mechanism employed by the affected cell (Kovářová et al., 2012). For instance, despite the lack of *SURF1* protein, different degrees of residual assembly and function of RC IV have been observed in different tissues and animal models, pointing towards a variable dispensability of *SURF1* protein in the assembly of RC IV and the possibility of compensatory mechanisms that replace the function of the missing protein in certain cases (Brischigliaro & Zeviani, 2021).

Given the high tissue-specific dispensability of SURF1 protein in RC IV assembly and the variable cellular response to specific mitochondrial insults, it is imperative to study the molecular consequences of *SURF1* mutations in a relevant cellular context for the disease. Mutations in *SURF1* are most often associated with severe neurological impairment, particularly affecting the brain basal ganglia (Böhm et al., 2006; Ruhoy & Saneto, 2014). Neurons, especially in regions with high energy demands such as the basal ganglia, may become more vulnerable to mitochondrial dysfunction, potentially leading to selective neurodegeneration that gives rise to the symptomatology of LS caused by *SURF1* mutations.

1.3.2. Neuropathology and biochemistry of *SURF1*-LS

The diagnosis of LS was initially based on the presence of *post-mortem* neuropathological lesions consisting of bilateral subacute necrotizing encephalomyopathy (Leigh, 1951). *Pre-mortem* diagnosis became feasible only with the advent of computed tomography and magnetic resonance imaging (MRI). Neuroimaging of LS is characterized by symmetric, focal necrotic lesions - observed as hyperintense lesions on T2-weighted imaging in MRI - predominantly affecting the basal ganglia (Bonfante et al., 2016; Distelmaier & Klopstock, 2023). These symmetric lesions are an identifying characteristic of the disease. Still, significant interpatient heterogeneity exists regarding lesion distribution and severity of the lesions.

Identified as one of the most frequent causes of LS, defects in *SURF1* lead to impaired RC IV assembly and consequent ATP depletion (Tiranti et al., 1998). Clinically, patients with *SURF1* mutations frequently present with lesions at the basal ganglia and the midbrain (Bonfante et al., 2016; Kose et al., 2020; Wedatilake et al., 2013). Additional involvement of other brain regions such as the thalamus or the brainstem may occur in some cases (Bonfante et al., 2016; Kose et al., 2020; Wedatilake et al., 2013).

Histologically, these brain lesions are characterized by necrosis accompanied by demyelination and gliosis, with some areas of relative neuronal preservation (Lake et al., 2015). This pattern of partial neuronal preservation within areas of significant neurodegeneration is a remarkable feature of LS. Although the functional status of these preserved neurons remains unclear, histopathological reports suggest a selective neuronal vulnerability in the basal ganglia and midbrain regions in patients with *SURF1* mutations (Lake et al., 2015).

Beyond these neuropathological features, certain alterations are hallmarks of LS (Rahman, 2023). Patients with *SURF1* mutations commonly present metabolic acidosis and elevated lactate levels in blood, urine, and cerebrospinal fluid (Kose et al., 2020; Stenton et al., 2022; Wedatilake et al., 2013). Their histochemical studies frequently show RC IV deficiency, with increased lipid accumulation and type I fiber predominance in muscle biopsies. Moreover, ultrastructural analysis of patient samples reveals the presence of

swollen mitochondria with abnormal mitochondrial cristae, although biopsies and ultrastructural studies are often not required for diagnosis (Kose et al., 2020; Stenton et al., 2022; Wedatilake et al., 2013).

The high inter-patient variability in LS complicates the diagnosis and the discovery of disease-modifying treatments for this mitochondrial disorder. Despite these challenges, the biochemical and neuropathological features of LS provide critical insights to develop representative models of the disease. However, understanding the key molecular mechanisms driving the disease pathology are essential to find effective therapeutical interventions.

1.3.3. Animal models of *SURF1* gene defects

Several *in vivo* animal models have been generated to study the alterations caused by the lack of SURF1 protein (summarized in Table 1). The first animal model ever generated was the *Surf1* knockout mouse (*Surf1*^{KO}) (Agostino et al., 2003). Here, the transduction of *Surf1* gene was disrupted by inserting a neomycin cassette and replacing exons 5 to 7. This knockout model led to embryonic lethality in most mice, probably due to the high toxicity of the neomycin cassette rather than being an actual effect of the mutation. The surviving animals showed a deficiency in RC IV activity only in selected tissues, such as the liver and muscle, but not the heart or the brain (Agostino et al., 2003). Consequently, these mice had altered motor skills but did not develop any neuropathology.

The second mouse model targeted exon 7 of the *Surf1* gene. These *Surf1*^{KO} mice showed a prolonged lifespan with no signs of neuropathology despite presenting reduced RC IV activity in the brain and exhibiting metabolic alterations such as higher glucose utilization and increased lactate production (Dell'Agnello et al., 2007; Lin et al., 2013). In fact, these *Surf1*^{KO} mice were resistant to neurodegeneration upon treatment with kainic acid, a seizure-causing agent. Kainic acid is a glutamate receptor agonist that induces calcium-mediated excitotoxicity and neuronal death in wild-type mice. Nevertheless, primary neurons of *Surf1*^{KO} mice exhibited higher mitochondrial calcium buffering capacity that protected them against the calcium-mediated neurotoxic effects of kainic acid, conferring neuroprotection against this insult (Lin et al., 2013).

Despite presenting mitochondrial alterations (Table 1), *Surf1*^{KO} mice developed normally, besides presenting mild motor deficits (Dell'Agnello et al., 2007) and decreased adiposity (Deepa et al., 2013). In these mice, RC IV dysfunction prompted a shift in substrate utilization (such as increased fatty acid oxidation) and metabolic changes (such as altered insulin sensitivity) that may be linked to the prolonged lifespan (Deepa et al., 2013).

A prolonged lifespan was also observed in *C. elegans* and a *Drosophila* model with specific *Surf1* knockdown (KD) in the central nervous system (CNS). Targeted silencing of *sft-1* (the SURF1 homologue in *C. elegans*) by RNA interference produced a model with deficiencies in RC IV activity that led to increased longevity, which was also linked to the insulin pathway

(Maxwell et al., 2013). These nematodes did not present any neuromuscular problems and developed normally, only exhibiting reduced reproductive capacity, likely due to distinct species-specific RC IV distribution and energy utilization (Maxwell et al., 2013)

Drosophila harboring CNS-specific *Surf1* KD (*Surf1*^{CNS-KD}) exhibited reduced RC IV activity in the brain, resulting in a prolonged lifespan with abnormal development of visual pathways but with no signs of neurodegeneration (Zordan et al., 2006). Instead, ubiquitous and muscle-specific KD of *Surf1* in *drosophila* models showed 100% egg-to-adult lethality, highlighting the distinct tissue-specific dependency on RC IV activity (Da-Rè et al., 2014; Zordan et al., 2006).

The tissue-specific dependency on COX activity may vary between species, giving rise to heterogeneous phenotypes. For instance, while *Surf1*^{KO} mice and *C.elegans* developed normally, CNS-restricted *Surf1* defects in *drosophila* and ubiquitous *Surf1* deficiency in two different zebrafish models led to visual abnormalities (Table 1).

The first zebrafish model was created using antisense morpholino oligonucleotides, which allowed for a dose-dependent loss of function by impeding the translation of the *Surf1* homologue in these animals (Baden et al., 2007). Using this model, zebrafish developed an abnormal head shape and microphthalmia with reduced swimming capacity and a strong cardiovascular pathology that eventually led to death. In addition, severe impairment of the development of secondary motor neurons, with the absence of axons and a reduced number of neuronal cell bodies was reported (Baden et al., 2007).

A more recent model in zebrafish using CRISPR-mediated KD showed reduced RC IV protein expression and activity with increased lactate level in tissue with no significant neuropathology (Haroon et al., 2023). Instead, these animals exhibited some eye abnormalities with respiratory defects and exercise intolerance, while neurodegeneration was only observed upon treatment with RC IV inhibitor sodium azide (Haroon et al., 2023).

Finally, given the lack of success of small animal models in faithfully reproducing the neuropathological signs observed in patients with *SURF1* mutations, a bigger animal model was generated by knocking down *SURF1* gene in pigs (Quadalti et al., 2018). *SURF1*^{KO} piglets presented high lethality with severe phenotype at birth, with animals having difficulties in swallowing, presenting poor body weight gain, and failure to thrive (Table 1). They exhibited a general reduction of mitochondrial ETC complexes in skeletal muscle with impaired motor response, but no significant difference in RC IV activity in the brain or in lactate in blood. At the neuropathological level, *SURF1*^{KO} piglets did not present any gross neurodegeneration but exhibited a reduction in cortical thickness with cytoarchitectural defects and increased presence of immature neurons (Quadalti et al., 2018).

ANIMAL MODEL	CLINICAL PHENOTYPE	BIOCHEMICAL AND MOLECULAR FEATURES	NEUROPATHOLOGY
PIG (Quadalti et al., 2018)	▪ ↓ Lifespan ▪ Failure to thrive ▪ Impaired reflexes (suckling or rooting) ▪ Facial dysmorphism ▪ Respiratory problems ▪ Muscle weakness	▪ ↓ COX activity (<i>M</i>) ▪ ↓ RC expression and function (<i>M</i>) ▪ ↓ CIV-containing supercomplex (<i>M</i>) ▪ Ø Accumulation of assembly intermediates (<i>M</i>)	▪ ↓ Cortical thickness ▪ Aberrant layering (↑immature neurons) ▪ Abnormal glia markers distribution ▪ Ø Neurodegeneration
MOUSE (Deepa et al., 2013; Dell'Agnello et al., 2007; Lin et al., 2013; Pulliam et al., 2014)	▪ ↑ Lifespan ▪ Mild motor defects ▪ ↓ Adiposity	▪ ↓ COX activity (<i>B,M, H</i>) ▪ ↓ RC expression and function (<i>M</i>) ▪ ↓ CIV-containing supercomplex ▪ Accumulation of assembly intermediates (<i>L</i>) ▪ = ATP levels (<i>B,M,H</i>) ▪ ↑ Lactate (<i>B,H</i>) ▪ ↑ Glucose metabolism (<i>B,H</i>) ▪ ↑ Fatty acid oxidation (<i>A</i>) ▪ = ROS (<i>M, H</i>) ▪ ↓ Mitochondrial Ca²⁺ uptake (<i>B</i>) ▪ ↓ MMP (<i>H</i>) ▪ ↑UPRmt (<i>M</i>) ▪ ↑ Biogenesis (<i>A, M</i>)	
ZEBRAFISH (Baden et al., 2007)	▪ Severe developmental defects ▪ Strong cardiovascular pathology ▪ Muscle weakness	▪ ↓ COX activity (50%) ▪ = ATP levels ▪ ↑ Lactate ▪ ↑ ROS	▪ Apoptotic cells in head region and neuronal tube ▪ Impair development of 2ry motor neurons (axon projection)
ZEBRAFISH (Haroon et al., 2023)	▪ Reduced swimming capacity ▪ Abnormal ocular morphology ▪ Exercise intolerance	▪ ↓ COX expression and activity (70-85%) ▪ Increased lactate ▪ Similar supercomplexes levels ▪ Normal ATP levels	

Table 1. Summary of animal models harboring *SURF1* mutations.

ANIMAL MODEL	CLINICAL PHENOTYPE	BIOCHEMICAL AND MOLECULAR FEATURES	NEUROPATHOLOGY
DROSO-PHILA (Zordan et al., 2006)	<i>Ubiquitous KD:</i> 100% egg-to-adult lethality - Altered locomotion <i>CNS-specific KD:</i> - Prolonged lifespan - Altered locomotion	<i>Ubiquitous KD:</i> - Altered mitochondrial morphology (M) <i>CNS-specific KD:</i> ↓ COX activity	- Ø Neurodegeneration - Abnormal visual pathways
DROSO-PHILA (Da-rè et al., 2014)	<i>Ubiquitous KD:</i> 100% egg-to-adult lethality - Altered locomotion <i>Muscle-specific KD:</i> 100% egg-to-adult lethality - Impaired muscle development	<i>Ubiquitous KD:</i> ↓ MRC activity - Transcriptomic downregulation of energy-related pathways <i>Muscle-specific KD – (M):</i> ↓ MRC activity ↓ Mitochondrial Ca²⁺ uptake - Altered mitochondrial morphology <i>CNS-specific KD – (B):</i> ↓ COX activity	
C.ELEGANS (Maxwell et al., 2013)	↑ Lifespan ↓ Germ cell production	↓ COX activity - Similar response to oxidative stress	

Table 1. Summary of animal models harboring *SURF1* mutations (continued). *SURF1* disease models (first column) with described clinical phenotypes, biochemical defects, and neuropathological alterations (when reported). Original publications for each animal model are referenced on the first column. Symbols and abbreviations: CNS: central nervous system; KD: knockdown; (B): brain; (M): muscle; (H): heart; (A): adipose tissue; ↑: increased, ↓: decreased; Ø: none; =: normal.

Altogether, the different animal models developed so far have failed to reproduce the pathological signs of LS in humans. In many of these animals, lack of *Surf1* leads to increased lifespan with no sign of neuronal pathology, while in others, it leads to embryonic lethality. These dramatic differences highlight the remarkable species-specific role of *Surf1* on RC IV assembly, despite being a highly conserved protein in eukaryotic species (Poyau et al., 1999).

The different dependency of *Surf1* on RC IV assembly, as well as the existence of species-specific redundant factors, may explain the different degrees of residual RC IV activity observed in different animals. For instance, *Surf1*^{KO} mice and piglets showed only 30-50% reduction of RC IV activity depending on the tissue (Dell'Agnello et al., 2007; Quadalti et al., 2018), while in humans, patients retain only 5-15% of COX activity (Tiranti et al., 1998). Thus, the inability to reduce RC IV activity below a sufficiently severe residual threshold may prevent animal models from reproducing the severity of the human phenotype.

These species-specific differences add complexity to the tissue-specificity of RC IV assembly (Kovářová et al., 2012, 2016). Different tissues may display different thresholds of tolerable RC IV impairment and cellular energy requirements, which could fluctuate and vary between species. For example, in nematodes, RC IV deficits were most prominent in the gonads since most of the energy is required for reproduction, but this is not seen in other animal models nor in humans.

Therefore, for the study of *SURF1* mutations, it is becoming imperative to assess the specific molecular pathomechanisms in a relevant cellular context and a species-specific manner. This thesis will focus on exploring the development of novel human models of LS, understanding how *SURF1* mutations contribute to the disease mechanisms, and on investigating potential therapeutic strategies.

1.3.4. Experimental therapies to treat LS

LS remains an incurable disease, with current treatments only providing supportive care to mitigate associated complications. For instance, antiepileptic drugs are used to manage seizures, while other symptomatic interventions may address respiratory, cardiovascular, and neuromuscular dysfunctions (Kornblum et al., 2023).

In the past decade, significant research efforts have accelerated the development of experimental therapies to treat LS (Bottani et al., 2020; Russell et al., 2020; Viscomi & Zeviani, 2023). Among the most promising strategies are gene therapy and pharmacological interventions aimed at modulating mitochondrial biogenesis and oxidative stress.

a) Gene therapy

A definitive cure for mitochondrial diseases like LS will likely come from gene therapy. Gene therapy has been a medical breakthrough for rare diseases like β -thalassemia, sickle cell disease, and Duchenne muscular dystrophy, several of which have received approval from the U.S. Food and Drug Administration (FDA).

In mitochondrial diseases, gene therapy or gene replacement therapy would restore normal protein function by delivering the wild-type form of the defective gene, effectively correcting the cellular problem (Di Donfrancesco et al., 2022). Adeno-associated viral (AAV) vectors are the leading delivery system due to their favorable safety profile, stable gene expression, and ability to transduce post-mitotic cells, including neurons (Wang et al., 2019).

However, many preclinical studies are still required to optimize safety and therapeutical efficacy before reaching the bedside (Corrà et al., 2022; Di Meo et al., 2017; Ling et al., 2021; Silva-Pinheiro et al., 2020). To achieve widespread clinical applicability, challenges such as immune responses, tissue targeting specificity, and the need for long-term expression must be addressed.

In the context of LS, a multisystemic disease, preclinical studies must determine the best route of administration, the optimal AAV serotype, and vector titration to reach effectiveness in the maximum number of cells (Bottani et al., 2020; Di Donfrancesco et al., 2022; Viscomi & Zeviani, 2023).

In the context of *SURF1* mutations, one promising study demonstrated that a single intrathecal administration of AAV serotype 9 (AAV9) vector carrying the human *SURF1* gene partially restored RC IV activity in a *Surf1*^{KO} mouse (Ling et al., 2021). However, the lack of pathological symptoms in this animal model limited the evaluation of this therapy's effectiveness.

Conventional gene therapy approaches can be employed for mutations in nuclear genes (e.g. *SURF1*, *NDUFS4*). However, mitochondrial diseases may face higher technical challenges for precise gene editing mtDNA (Tolle et al., 2023). New strategies such as the double-stranded DNA cytosine base editor (ddCBE) approach, together with mitochondrial-targeted zinc finger nucleases, TALENs and CRISPR-free base editing are under investigation with promising results in preclinical models (Cho et al., 2022; Gammage et al., 2018; Lee et al., 2023; Mok et al., 2020).

Beyond technical limitations, the clinical translation of gene therapy faces significant obstacles, including high production costs, regulatory requirements, and challenges in achieving widespread distribution to affected tissues, particularly the brain. Future advances in this therapeutic avenue will be crucial in overcoming these barriers and making gene therapy a viable option for LS patients.

b) Experimental pharmacological interventions

The growing recognition of mitochondria as central regulators of cellular signaling has paved the way for pharmacological approaches targeting key mechanisms disrupted by mitochondrial dysfunction. Two major strategies have emerged: enhancing mitochondrial biogenesis and mitigating oxidative stress.

Antioxidants for modulating oxidative stress

Antioxidant drugs offer potential benefits for various mitochondrial disorders. While they do not correct the underlying mitochondrial dysfunction, antioxidants can mitigate ROS-mediated toxicity and improve the overall cellular energy metabolism (Bottani et al., 2020; Viscomi & Zeviani, 2023).

Several antioxidant compounds- such as Sonlicromanol (De Haas et al., 2017; Klein Gunnewiek et al., 2021), Idebenone (Heitz et al., 2012; Morvan & Demidem, 2018), or N-acetyl cysteine (NAC) (Viscomi et al., 2010)- have shown promising results in preclinical models of mitochondrial diseases and are currently undergoing clinical trials (Karaa & Klopstock, 2023). Among these, Idebenone stands out as the only antioxidant therapy approved in the European Union for the treatment of Leber's hereditary optic neuropathy (LHON). Additionally, Sonlicromanol has gained recognition for its therapeutic potential, receiving an orphan drug designation (ODD) by the European agencies for the treatment of mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS) syndrome (EU/3/19/2168).

However, most antioxidant treatments tested in the context of LS have failed to demonstrate significant clinical efficacy (Viscomi & Zeviani, 2020). In addition to their limited benefits, these interventions blunt ROS indiscriminately and may interfere with the physiological role of ROS in cellular signaling (Lennicke & Cochemé, 2021; Shadel & Horvath, 2015) and differentiation (Chakrabarty & Chandel, 2021).

Activation of mitochondrial biogenesis

Mitochondrial diseases are characterized by defective ATP production, yet clinical symptoms often manifest upon metabolic stress when mitochondrial activity drops below a critical threshold. Stimulating mitochondrial biogenesis has been proposed as a therapeutic approach to enhance the number of functional mitochondria (Bottani et al., 2020). However, this strategy carries potential risks, including increased ROS production and mtDNA damage.

Mitochondrial biogenesis is controlled by a complex signaling cascade requiring coordination between nuclear and mitochondrial genomes (Popov, 2020). The master regulator of this process is PGC-1 α (*peroxisome proliferator-activated receptor gamma coactivator 1-alpha*), which integrates external and internal stimuli (e.g., cold, exercise, nutrition) to adjust mitochondrial mass according to cellular energy needs (Bottani et al., 2020). Preclinical models overexpressing PGC-1 α have demonstrated promising outcomes

in disease rescue. For example, PGC-1 α overexpression in *Surf1*^{KO} mice induced mitochondrial proliferation and increased RC IV activity in skeletal muscle (Viscomi et al., 2011). Similarly, in human neural models with *SURF1* mutations, PGC-1 α overexpression improved OXPHOS bioenergetics and neuronal morphology (Inak et al., 2021).

Given these findings, several pharmacological strategies are being developed to activate PGC-1 α and stimulate mitochondrial biogenesis (Figure 8). The regulation of PGC-1 α activity is highly complex, modulated by multiple post-translational modifications, including phosphorylation by AMP-activated protein kinase (AMPK) and deacetylation by deacetylation by sirtuin 1 (SIRT1), both of which represent potential pharmacological target (Popov, 2020).

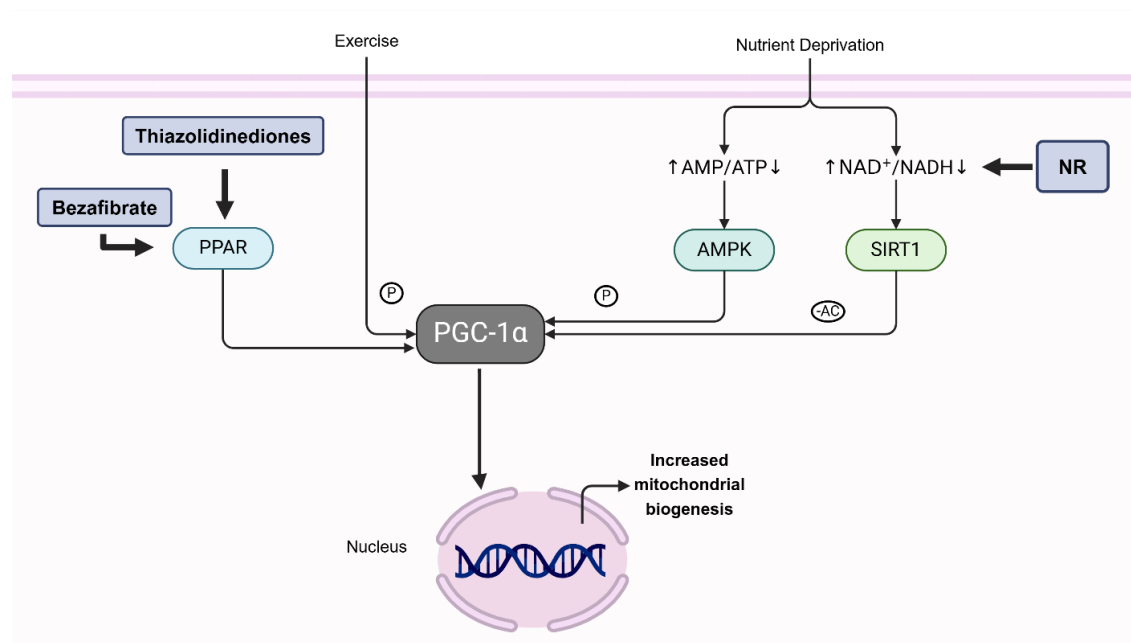


Figure 8. Schematic representation of pathways regulating mitochondrial biogenesis. External factors (exercise, caloric restriction) or drugs such as bezafibrate, thiazolidinediones, nicotinamide riboside (NR), and CBD upregulate the expression of PGC-1 α , which in turn activates essential mitochondrial genes. Black thick arrows indicate positive regulations, “P” indicates phosphorylations and “-AC”, deacetylations. Illustration created with Biorender, adapted from (Bottani et al., 2020).

The activation of PGC-1 α via SIRT1 deacetylation is a nicotinamide adenine dinucleotide (NAD⁺)-dependent process (Cantó et al., 2015). Mitochondrial dysfunction often results in NAD⁺ depletion and a reduced NAD⁺/NADH ratio, leading to pyruvate accumulation and excessive lactate production, a hallmark of mitochondrial disease (Russell et al., 2020). Supplementation with NAD⁺ precursors, such as nicotinamide riboside (NR), has been shown to enhance mitochondrial biogenesis via the SIRT1/PGC-1 α axis (Cantó et al., 2015). In preclinical models, NR supplementation improved motor performance in mitochondrial disease mouse models and has shown promising results in clinical trials for mitochondrial myopathies (Khan et al., 2014).

Another approach to modulating PGC-1 α involves targeting peroxisome proliferator-activated receptors (PPARs), a family of nuclear receptors that regulate lipid metabolism and mitochondrial function (Popov, 2020). This family includes three isoforms—PPAR α , PPAR β/δ , and PPAR γ —each with distinct physiological roles and tissue distributions (Mirza et al., 2019). Upon activation, the PPAR γ can form heterodimers with the retinoid X receptor (RXR) and bind to PPAR response elements (PPRE) in gene promoters, leading to transcriptional activation of metabolic genes, including PGC-1 α (Jamwal et al., 2021).

Overexpression of PGC-1 α in the skeletal muscle of *Surf1*^{KO} mouse showed robust induction of mitochondrial biogenesis and increased mitochondrial respiratory chain activities, including a rescue of RC IV activity (Viscomi et al., 2011).

Pharmacological induction of PGC-1 α was attempted using Bezafibrate, a pan-PPAR agonist, that initially showed promise in cell models of mitochondrial dysfunction. In these models, bezafibrate increased the mitochondrial ETC complex activities and improved ATP production (Bastin et al., 2008; Casarin et al., 2012; Douiev et al., 2020). In human neural models carrying *SURF1* mutations, bezafibrate treatment enhanced mitochondrial respiration, ATP production, mtDNA copy number, and neuronal morphology, significantly upregulating PGC-1 α expression (Inak et al., 2021).

However, preclinical *in vivo* studies yielded mixed results, failing to induce mitochondrial biogenesis (Viscomi et al., 2011; Yatsuga & Suomalainen, 2012). Additionally, a small clinical study revealed a significant metabolic imbalance that questions its long-term safety despite some improvements in muscle and cardiac function in patients (Steele et al., 2020).

An alternative strategy involves activating PPAR γ with thiazolidinediones. In the context of mitochondrial disease, Leriglitazone has been recently used in a *Ndufs4*^{KO} model effectively prolonging the lifespan and mitigating disease symptoms (Puighermanal et al., 2024). Notably, this study identified Cannabidiol (CBD) as a potential therapeutic agent, demonstrating its beneficial effects in a mouse model of mitochondrial RC I deficiency via partial activation of PPAR γ (Puighermanal et al., 2024). CBD has already obtained the ODD by the European regulatory agencies as adjunctive therapy for the treatment of certain pediatric forms of epilepsy occurring in Lennox-Gastaut syndrome or Dravet syndrome under the commercial name of Epidyolex (EMA/H/C/004675). Most recently, CBD obtained the ODD for the treatment of LS (EU/3/23/2800).

While both antioxidant therapies and mitochondrial biogenesis stimulation offer promising avenues for mitigating mitochondrial dysfunction, neither approach directly addresses the underlying genetic defect. Antioxidants can help alleviate oxidative stress and protect cellular components, while pharmacological induction of mitochondrial biogenesis may enhance energy production in compromised cells. However, further research is essential to refine these strategies, improve efficacy, and assess long-term safety.

1.4. DISEASE MODELING AND DRUG DISCOVERY USING INDUCED PLURIPOTENT STEM CELLS (iPSCs)

1.4.1. iPSC-derived neural models

In 2006, Shinya Yamanaka and Kazutoshi Takahashi discovered that the ectopic expression of four exogenous factors (OCT3/4, SOX2, KLF4 and c-MYC) enabled the reprogramming of somatic cells into induced pluripotent stem cells (iPSC) (Takahashi & Yamanaka, 2006). iPSCs have the capacity for self-renewal and differentiation and can be generated from human skin fibroblasts or other somatic cells, such as blood cells. Thus, this groundbreaking technology holds the power to study human-specific molecular processes in tissues that are otherwise difficult to access for disease modeling, such as the brain.

Since its discovery, there has been great advancement in the generation of human *in vitro* models to study the human brain, from two-dimensional (2D) neural progenitor cells (NPCs) and neurons to more complex three-dimensional (3D) brain organoids (Figure 9).

NPCs are lineage-restricted, mitotically active cells derived from stem cells. These multipotent stem cells are capable of differentiating into neurons, astrocytes, and oligodendrocytes while remaining proliferative, offering a scalable and cost-effective model for drug discovery applications (Heilker et al., 2014).

In the context of mitochondrial dysfunction, NPCs have been proposed to be a key cellular type for disease modeling and drug discovery. Mitochondria within NPCs acquire an elongated morphology with defined cristae and dense matrices, a morphological change that is accompanied by a metabolic shift towards OXPHOS metabolism that is impaired in mitochondrial diseases (Brunetti et al., 2021). Thus, NPCs acquire morpho-functional characteristics that resemble mitochondria of differentiated iPSC-derived neurons while holding a proliferative phenotype (Lorenz et al., 2017).

The efficient generation of NPCs from iPSCs can be achieved using a combination of small molecules based on dual SMAD inhibition. The dual SMAD inhibition is based on the use of bone morphogenic protein (BMP) and transforming growth factor beta (TGF β) inhibitors that drive the formation of early NPCs within 10 days, leading to relevant neuronal subtypes within 20 days (Reinhardt et al., 2013).

Nevertheless, the use of small molecules gives rise to a mixed neuronal culture with little control over the exact cellular composition. To overcome this high variability, directed neuronal differentiation protocols that induce specific neural lineages were developed to ensure a more homogeneous cell population. An example of this approach is the protocol developed by Zhang et al. in 2013 based on the overexpression of Neurogenin 2 (NGN2)

transcription factor to obtain a highly homogeneous population of glutamatergic excitatory neurons from iPSCs (Zhang et al., 2013).

Overexpression of *NGN2* has proven to be a highly efficient and cost-effective approach for generating induced neurons in under two weeks (Zhang et al., 2013). *NGN2*, a master regulator of neurogenesis, allows the generation of neurons from fibroblast, iPSC, neural progenitors, and even the trans-differentiation of glial cells with high reproducibility (Hulme et al., 2022). Its robustness and easy-to-use approach have made iNs widely used in neurodevelopmental and disease modeling, high-throughput drug screening, and neuron replacement therapies (Hulme et al., 2022).

Additionally, the differentiation of specific neuronal subtypes (dopaminergic, inhibitory) as well as astrocytes and oligodendrocytes from human iPSCs has also been possible using transcription factor-based and small molecules-based approaches with different degrees of efficiency (Caiazzo et al., 2011; Canals et al., 2018; Tanabe et al., 2022).

While 2D models have been instrumental in studying neural differentiation, they fail to capture the cellular and spatiotemporal development of the structural complexity of the brain, leading to the generation of 3D organoid models. Lancaster et al were the first to develop a protocol to generate self-organize 3D brain organoids from iPSC without the use of growth factors. This protocol enabled the generation of a wide variety of brain regions representative of the dorsal cortex, ventral forebrain, hippocampus, choroid plexus, and retina (Lancaster et al., 2013).

Brain organoids exhibit a similar gene expression profile and epigenomic signatures when compared to fetal brains, even recapitulating the timing of human neurodevelopment *in vitro* (Camp et al., 2015; Cheroni et al., 2022). Therefore, brain organoids have become useful models for studying human neurodevelopmental processes such as early neurogenesis, species-specific spatiotemporal differences, or achieving a better understanding of the effect of specific mutations on human neurodevelopment (Paşca, 2018). For instance, in the case of autism spectrum disorder (ASD), brain organoids have been used to understand key developmental differences in cases of idiopathic ASD (Jourdon et al., 2023; Mariani et al., 2015; Paulsen et al., 2022; Pigoni et al., 2023) and more recently to screen for genetic risk genes and create a gene regulatory network (Li et al., 2023). In addition, combined with functional readouts such as electrophysiological recordings or calcium analysis, organoids can shed some light on the functional imbalances occurring in ASD (Paulsen et al., 2022).

The study of neurodevelopmental disorders using iPSC-derived models has underscored the importance of the human genomic context in fine-regulating certain processes (Paulsen et al., 2022). While phenotypic trends remain consistent across cell lines, their expressivity can be influenced by the individual genomic context. Therefore, these studies highlight the need for the use of isogenic lines when modeling neural disorders.

Isogenic human iPSC models, which share the same genetic background, provide a powerful tool for dissecting disease mechanism. By introducing or correcting mutations in an identical genomic context, these models allow for precise genotype-phenotype correlations, reducing variability and improving reproducibility. Therefore, when modeling neural disorders, it is essential to consider this fact to properly design the modeling strategies and not only use engineered cell lines in a control background but also add the complexity of using patient-derived cell lines.

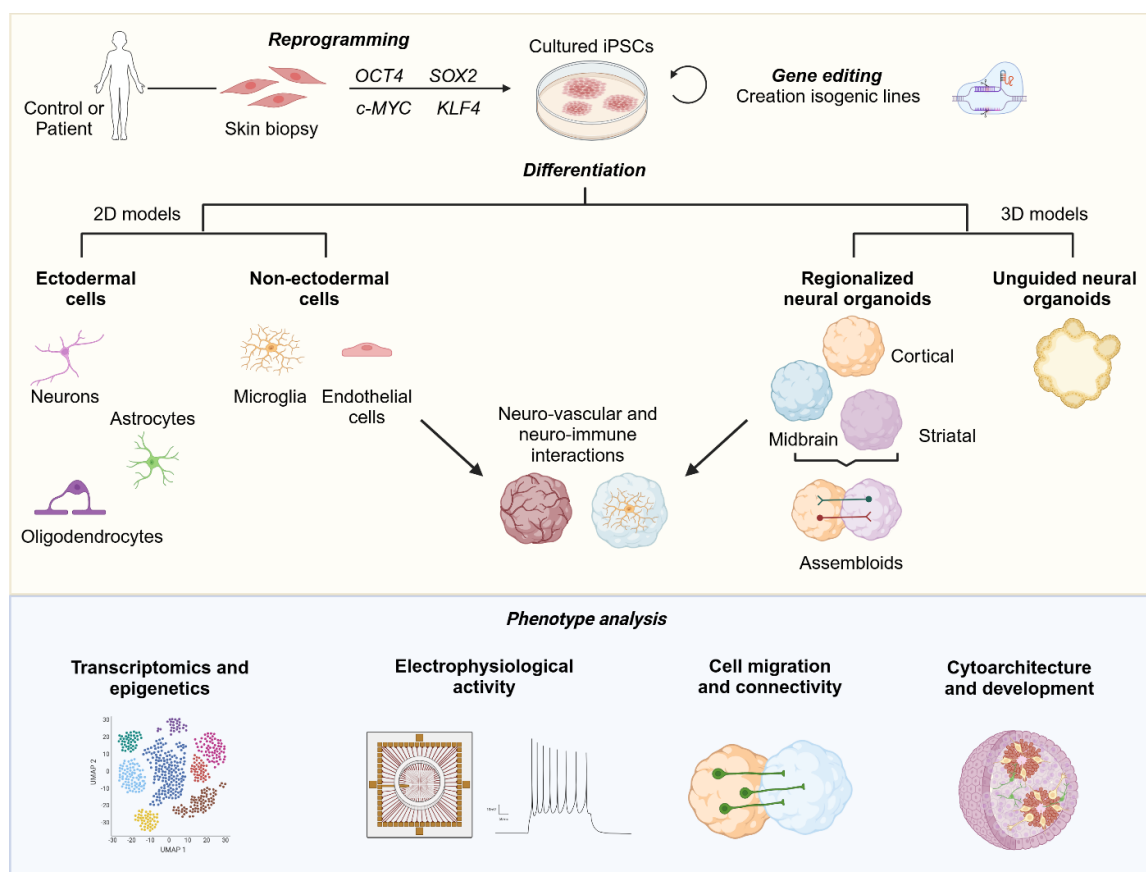


Figure 9. Brain Organoid Based Models to Identify Human-Specific Processes of Brain Development.

Scheme outlining methods for establishing iPSC-based neural models and downstream analysis to compare resulting phenotypes. Control and patient-derived skin samples can be used to generate reprogrammed induced pluripotent stem cells (iPSCs), which can then be genetically engineered to obtain isogenic lines. Human iPSCs can be differentiated into different cell types from the ectodermal layer (such as neurons, astrocytes and oligodendrocytes) as well as other cell types present in the human brain with different embryonic origins, including microglia or endothelial cells. Three-dimensional (3D) unguided and regional-specific neural organoids can be produced from iPSCs and be further combined into assembloids to observe neuronal migratory patterns and brain connectivity. Integration of non-ectodermal cell types into brain organoids elevates the biological relevance of the system, allowing the study of neuro-vascular and neuro-immune interactions that are crucial for certain diseases. Resulting organoids are useful tools to study human specific phenotypes, including complex, functional assays such as micro-electrode arrays (MEA) cytoarchitectural differences during development or differentially expressed genes with high-throughput omic analyses like single-cell RNA-sequencing. Illustration created with Biorender.

Brain organoids hold immense potential to study human brain development and disease; however, they currently face some limitations that the field is rapidly evolving to overcome. The generation of brain organoids is highly variable, time-consuming, and requires expertise. Efforts to enhance the robustness and reproducibility of the system have led to the development of more guided protocols for generating regionalized brain organoids (Kelley & Paşca, 2022).

Generation of regionalized human organoids resembling the cerebral cortex (Mariani et al., 2015; Paşca et al., 2015; Qian et al., 2016), striatum (Miura et al., 2020), cerebellum (Muguruma et al., 2015) or midbrain (Jo et al., 2016; Monzel et al., 2017; Qian et al., 2016) regions has been possible using bath application of morphogens that achieve a similar cellular composition of the target tissue. Whilst this improves reproducibility, it reduces the overall complexity of the system. To address this, different regionalized organoids can be assembled into so-called “assembloids” to obtain a complex system with different neuronal subtypes that allow the study of functional networks (Onesto et al., 2024).

Despite their increased complexity, brain organoids are still an imperfect system. Brain organoids are obtained by directing iPSC differentiation towards the neuroectodermal lineage, and then further differentiated to follow a trajectory of brain development. Being of non-ectodermal lineage, microglia and endothelial cells are key cellular components in the human brain that are lacking in this *in vitro* model (Benito-Kwiecinski & Lancaster, 2020). To circumvent this limitation, several groups have developed co-culture systems incorporating iPSC-derived microglia into brain organoids (Abud et al., 2017) or have utilized microfluidic chips to recreate the nutrient flow (Cakir et al., 2019). These ongoing advancements will likely continue to refine iPSC-derived models, bringing them closer to recapitulating the complexity of the human brain in both health and disease (Pagliaro et al., 2025).

1.4.2. iPSC-derived neural models to study LS and HD

Mitochondria play a central role in neuronal function by supplying energy for synaptic activity, regulating calcium homeostasis, or modulating oxidative stress responses, among other functions (Picard & Shirihi, 2022; Rangaraju et al., 2019). Additionally, recent investigations underscore the role of this organelle in neuronal fate commitment and morphogenesis via metabolic programming, strategic spatiotemporal distribution, and morphodynamical changes (Iwata & Vanderhaeghen, 2021; Khacho et al., 2019).

During neuronal development, mitochondria undergo a series of morpho-metabolic changes in the transition from neural stem cells towards highly specialized neurons (Khacho et al., 2019). Neural stem cells are highly glycolytic cells to sustain a high degree of proliferation. After division, daughter stem cells destined to become neurons undergo mitochondrial fission, with newborn neurons having small and fragmented mitochondria that grow gradually into their classic tubular shape and acquire an oxidative profile during

neuronal maturation (Lisowski et al., 2018). This crucial metabolic development sets the tempo of neuronal maturation and exhibits a species-specific pattern (Iwata, 2022; Iwata & Vanderhaeghen, 2021).

The generation of neurons from patient-derived iPSC allows the investigation of how mitochondrial alterations impact neuronal differentiation, morphology, and function in a human genomic and cellular context. Additionally, in the case of mitochondrial diseases, traditional cellular and animal models often fail to fully recapitulate human mitochondrial physiology due to species-specific differences in mtDNA regulation, bioenergetics, and metabolic interactions.

In the context of LS, several studies have studied the impact of mitochondrial mutations using iPSC-derived neurons and brain organoids (Heiduschka & Prigione, 2025; Henke et al., 2024). Cerebral organoids carrying *SURF1* mutations displayed aberrant neuronal morphogenesis that stem at the level of NPCs, which failed to metabolically rewire to OXPHOS and retain a glycolytic, proliferative state that impeded their commitment towards neuronal fate (Inak et al., 2021).

NPCs carrying *NDUFS4* mutations, affecting complex I, showed reduced neurite outgrowth and decreased MMP (Inak et al., 2021). In brain organoids, *NDUFS4* mutations led to upregulation of proinflammatory pathways, where neurons appeared more susceptible to glutamate-mediated excitotoxicity (Daneshgar et al., 2023), in agreement with increased neuronal apoptosis observed in another study (Yoon et al., 2022). Similarly, complex I with a defective *NDUFV1* gene displayed decreased ATP production and MMP in NPCs and neurons, with increased ROS levels (Sequiera et al., 2020).

Affected respiratory-linked ATP production, dysregulated MMP, and altered calcium homeostasis have also been described in iPSC-derived NPCs and neurons harboring mtDNA mutations in complex I (*MT-ND5*) and complex V (*MT-ATP6*) which are known causes leading to Maternally-Inherited Leigh Syndrome (MILS) (Galera-Monge et al., 2020; Lorenz et al., 2017; Zheng et al., 2016). Additionally, *MT-ATP6* mutations in iPSC-derived cerebral organoids displayed defective cortical cyto-architecture with affected neuroepithelial bud generation (Romero-Morales et al., 2022), demonstrating the tight link between mitochondrial dysfunction and faulty neurodevelopment.

In the context of HD, normal HTT protein has been involved in neuronal development and survival (Reiner et al., 2003). Accordingly, it was shown that mHTT interfered with human neurodevelopmental processes in human fetuses, affecting neuronal progenitor cell polarity and differentiation, among other features (Barnat et al., 2020).

Similarly, iPSC-derived NPCs and neurons from HD patients displayed neurodevelopmental alterations, including abnormal neural rosette formation, altered axon guidance, and disrupted calcium signaling (Kaye et al., 2022). These models also revealed metabolic alterations, such as changes in mitochondria number, density, and respiration defects (Kaye et al., 2022; Kedaigle et al., 2021). Additionally, these neurons

exhibited impaired morphology, maturation, and electrophysiological activity (Barnat et al., 2020; Mattis et al., 2012). Importantly, the severity of these disease-associated phenotypes was linked to the CAG repeat length and may be attenuated or absent when utilizing isogenic lines, highlighting the importance of using genetically modified iPSC lines to study HD-specific phenotypes.

Overall, mounting evidence indicates that both LS and HD exhibit a neurodevelopmental component of the disease that has been underexplored. These early alterations - ranging from defective neuronal commitment to impaired morphogenesis, maturation, and faulty network formation - may contribute to the onset and progression of the disease.

While iPSC-derived neural models uniquely allow capturing these early developmental stages in a species- and patient-specific manner, much of the current data has been generated using general, non-region-specific brain organoid protocols. This limits the anatomical relevance of the findings, particularly given the regional vulnerability observed in LS and HD—such as in the midbrain and basal ganglia. The implementation of regionally patterned brain organoids, including midbrain organoids (MOs), is therefore crucial.

MOs have already proven instrumental in modeling Parkinson's disease (PD), a neurodegenerative disorder characterized by the loss of dopaminergic neurons of the substantia nigra. In the context of PD, MOs have reliably recapitulated key features of midbrain development and dopaminergic neuron vulnerability (Smits et al., 2019; Zagare et al., 2022). Applying these models to LS and HD may enhance our understanding of disease pathogenesis in affected brain regions and facilitate the discovery of more targeted therapeutic strategies.

1.4.3. iPSC-derived neural models for drug discovery in mitochondrial disorders

The discovery of disease-modifying compounds is a demanding and costly process that often fails to successfully transition from preclinical models into the clinic, partly due to the poor predictivity of preclinical biological models used in drug discovery (Kola & Landis, 2004).

Traditionally, drug discovery has relied on target-directed approaches in heterologous recombinant systems, followed by validation in primary animal cells or *in vivo* animal models. Target-directed approaches offer a great artificial *in vitro* screening system with a high signal-to-noise ratio that efficiently identifies drugs that bind to the target of interest and remains valuable for diseases depending on a single molecular target. However, this approach lacks the cellular context and the species-specific differences of the target tissue, omitting interactions with physiologically relevant proteins and metabolites that may change the action of the compound (Heilker et al., 2014).

Given the complexity of neurological disorders and the challenge of testing candidate drugs against human primary neurons, iPSC-derived neural models provide a powerful alternative for compound validation in phenotype-based assays (Grskovic et al., 2011). Unlike target-directed approaches, phenotype-based assays focus on a pathologically relevant event rather than a specific molecular target, emphasizing measurable functional outcomes (Heilker et al., 2014).

Therapeutic advances for treating LS have been based on theoretical frameworks of mitochondrial dysfunction, which have ultimately failed in clinical trials due to the poor predictive power of preclinical biological models used in drug discovery. The emergence of stem cell models for mitochondrial diseases has led to the identification of early neuronal dysfunction in the disease, opening a new time window for potential therapeutic intervention (Brunetti et al., 2021; Marín, 2016).

In the case of LS, iPSCs have been used to validate the efficacy of different pharmacological strategies in ameliorating distinct disease phenotypes, such as neuronal survival or mitochondrial respiration (Heiduschka & Prigione, 2025; Inak et al., 2017). For example, iPSC-derived neurons have been used to validate the therapeutic effects of Idebenone, PGC-1 α overexpression, and NR supplementation in apoptosis assays in mitochondrial diseases (Danese et al., 2022; Yoon et al., 2022)

Similarly, bezafibrate was tested in *SURF1* iPSC-derived NPCs to enhance mitochondrial biogenesis, improving neuronal outgrowth and morphology (Inak et al., 2021). Rapamycin, which successfully rescued disease-relevant phenotypes in a complex I-deficient mouse model of LS, was validated in *MT-ATP6* iPSC-derived midbrain dopaminergic neurons, where it improved ATP production and reduced glutamate excitotoxicity (Zheng et al., 2016). Furthermore, using iPSC-derived *NGN2* neurons carrying the m.3243A>G mutation, Nael Kasri's group demonstrated that the antioxidant Sonlicromanol could partially restore neuronal network activity in MELAS (Gunnewiek et al., 2021). These studies illustrate how iPSC-based assays provide preclinical validation of compounds in a human model, facilitating the translatability of these compounds into the clinic.

In addition to validation, phenotype-based assays can be integrated into high-content imaging (HCI) pipelines, which combine automated microscopy with quantitative image analysis to assess how hundreds to thousands of compounds affect disease-related cellular traits in parallel. In a notable example, iPSC-derived NPCs carrying an *MT-ATP6* mutation were subjected to a phenotypic screen, a strategy in which compounds are selected based on their ability to restore a specific cellular phenotype. Using a library of FDA-approved compounds, the screen identified Avanafil, a phosphodiesterase type 5 (PDE5) inhibitor, as a candidate capable of rescuing MMP deficits observed in *MT-ATP6* NPCs (Lorenz et al., 2017). These findings led to the obtention of ODD status to the PDE5 inhibitor Sildenafil (EU/3/23/2831), highlighting the potential of phenotype-based screening to uncover novel therapeutic strategies beyond conventional approaches, which have often failed to translate into clinical success.

However, performing automated HCI-based compound screens using iPSC-derived neural models remains expensive and requires specialized infrastructure, limiting its accessibility. The high cost of cell culture, differentiation protocols, and imaging technologies create barriers to its widespread implementation, particularly in smaller research institutions or early-stage drug development programs.

To overcome these challenges, *in silico* screening approaches offer a promising complementary strategy to reduce costs, enhance efficiency, and improve predictive accuracy. Computational models generated using deep learning or machine learning can leverage large-scale biological datasets—including omics data, chemical libraries, and imaging-based phenotypic screens—to recognize intricate patterns between molecular structures and biological activity. These methods may be able to predict drug-target interactions, optimize lead compounds, and prioritize candidate compounds before validation in more complex models. The combination of *in silico* modeling with experimental validations in human iPSC-derived neural models holds great promise for accelerating drug discovery in neuroscience by bridging the gap between target-based and phenotype-driven approaches.

Overall, using human iPSC models in phenotype-based screening would help bridge the translational gap from preclinical studies into effective treatments for human neurological disorders. Future advancements in high-content multiplexed assays and integration of deep learning algorithms with iPSC-derived neural-based assays will facilitate automated, unbiased identification of novel drug responses, accelerating the discovery of effective therapeutics for mitochondrial disorders.

2. FRAMEWORK AND AIMS OF THIS THESIS



Mitochondrial dysfunction is a hallmark of neurodegenerative diseases such as HD and LS. Currently, there are no disease-modifying therapies to halt or reverse these diseases' progression, and only symptomatic treatments are available.

The discovery of effective treatments for these and other neurological diseases is partially hindered by the inaccessibility of primary tissue, the poor predictability of preclinical models, and significant species-specific differences in disease pathology. In this context, stem cell-derived neural cells emerge as a powerful tool for drug testing and disease modeling in neuroscience.

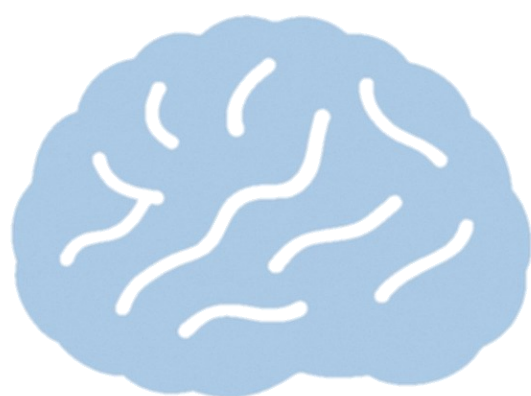
In this thesis, I aim to employ iPSC-derived neural models as a versatile tool to model how mitochondrial defects can affect neuronal morphogenesis in the context of LS and HD

In HD, mHTT leads to mitochondrial dysfunctions early in the disease, but the molecular mechanisms linking these defects to altered neuronal development have not been elucidated.

For LS, a primary mitochondrial disease, mutations in the *SURF1* gene cause RC IV dysfunction and lead to defective neural progenitor programming, ultimately affecting neuronal morphogenesis. The working hypothesis of this thesis is that defective neuronal morphogenesis can be a targetable disease phenotype to find new pharmacological treatments for LS.

For this, I will undertake the following objectives:

1. To develop a high-content imaging analysis tool to assess neuronal morphogenesis defects.
2. To establish a disease-relevant 3D model to study defective neuromorphogenesis linked to mitochondrial dysfunction in HD and LS.
3. To discover and validate new pharmacological strategies for the treatment of LS.
4. To describe the possible mechanisms of action of these new potential drugs.



3. RESULTS

3.1. GENERATION OF HUMAN BRAIN ORGANIDS FOR MITOCHONDRIAL DISEASE MODELING

Stephanie Le, Laura Petersilie, Gizem Inak, **Carmen Menacho-Pando**, Karl W. Kafitz, Agnieszka Rybak-Wolf, Nikolaus Rajewsky, Christine R. Rose, Alessandro Prigione.

Abstract

Mitochondrial diseases represent the largest class of inborn errors of metabolism and are currently incurable. These diseases cause neurodevelopmental defects whose underlying mechanisms remain to be elucidated. A major roadblock is the lack of effective models recapitulating the early-onset neuronal impairment seen in the patients. Advances in the technology of induced pluripotent stem cells (iPSCs) enable the generation of three-dimensional (3D) brain organoids that can be used to investigate the impact of diseases on the development and organization of the nervous system. Researchers, including these authors, have recently introduced human brain organoids to model mitochondrial disorders. This paper reports a detailed protocol for the robust generation of human iPSC-derived brain organoids and their use in mitochondrial bioenergetic profiling and imaging analyses. These experiments will allow the use of brain organoids to investigate metabolic and developmental dysfunctions and may provide crucial information to dissect the neuronal pathology of mitochondrial diseases.

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Generation of Human Brain Organoids for Mitochondrial Disease Modeling

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Introduction

Mitochondrial diseases represent the largest class of inborn errors of metabolism¹. They are caused by genetic mutations disrupting different mitochondrial processes, including oxidative phosphorylation (OXPHOS)², respiratory chain assembly, mitochondrial dynamics, and mitochondrial DNA transcription or replication³. Tissues with energy requirements are particularly affected by

mitochondrial dysfunction⁴. Accordingly, patients with mitochondrial diseases typically develop early-onset neurological manifestations.

There are currently no treatments available for children affected with mitochondrial diseases⁵. A major hindrance for drug development of mitochondrial diseases is the lack of



effective models recapitulating the human disease course⁶. Several of the currently studied animal models do not exhibit the neurological defects present in the patients⁷. Hence, the mechanisms underlying the neuronal pathology of mitochondrial diseases are still not fully understood.

Recent studies generated iPSCs from patients affected by mitochondrial diseases and used these cells to obtain patient-specific neuronal cells. For example, genetic defects associated with the mitochondrial disease, Leigh syndrome, have been found to cause aberrations in cellular bioenergetics^{8,9}, protein synthesis¹⁰, and calcium homeostasis^{9,11}. These reports provided important mechanistic clues on the neuronal impairment occurring in mitochondrial diseases, paving the way for drug discovery for these incurable diseases¹².

Two-dimensional (2D) cultures, however, do not enable the investigation of the architectural complexity and regional organization of 3D organs¹³. To this end, the use of 3D brain organoids derived from patient-specific iPSCs¹⁴ may allow researchers to gain additional important information and thereby help to dissect how mitochondrial diseases impact the development and function of the nervous system¹⁵. Studies employing iPSC-derived brain organoids to investigate mitochondrial diseases are beginning to uncover the neurodevelopmental components of mitochondrial diseases.

Spinal cord organoids carrying mutations associated with the mitochondrial disease, mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes syndrome (MELAS), showed defective neurogenesis and delayed motor neuron differentiation¹⁶. Cortical organoids derived from patients with the mitochondrial disease, Leigh syndrome, showed reduced size, defects in neural epithelial bud generation, and loss of cortical architecture¹⁷. Brain organoids from Leigh

syndrome patients showed that the disease defects initiate at the level of neural progenitor cells, which cannot commit to mitochondrial metabolism, causing aberrant neuronal branching and morphogenesis¹⁸. Thus, neural progenitors may represent a cellular therapeutic target for mitochondrial diseases, and strategies promoting their mitochondrial function may support the functional development of the nervous system.

The use of brain organoids might help uncover the neurodevelopmental components of mitochondrial diseases. Mitochondrial diseases are mainly considered as early-onset neurodegeneration⁵. However, neurodevelopmental defects are also present in patients affected by mitochondrial diseases, including developmental delay and cognitive impairment¹⁹. Patient-specific brain organoids may help address these aspects and elucidate how mitochondrial diseases may impact human brain development. Mitochondrial dysfunction could also play a pathogenetic role in other more common neurological diseases, such as Alzheimer's disease, Parkinson's disease, and Huntington's disease⁴. Hence, elucidating the impact of mitochondrial defects in neurodevelopment using brain organoids might also be instrumental for the study of those diseases. This paper describes a detailed protocol for generating reproducible brain organoids that can be used for conducting disease modeling of mitochondrial diseases.

Protocol

NOTE: The use of human iPSCs may require an ethical approval. iPSCs used in this study were derived from healthy control individuals following local ethical approval (#2019-681). All cell culture procedures must be performed under a sterile cell culture hood, carefully disinfecting all reagents and consumables before transferring under



the hood. Human iPSCs used for differentiation should have a passage number below 50 to avoid potential genomic aberrations that may occur upon extensive culture. The pluripotent state of the cells should be validated before organoid generation, for example, by monitoring the expression of pluripotency-associated markers such as NANOG or OCT4. Mycoplasma tests should be conducted weekly to ensure mycoplasma-free cultures.

1. Generation of brain organoids

1. Culture of human iPSCs

1. Culture human iPSCs under feeder-free conditions in iPSC medium (see the **Table of Materials**) on coated 6-well plates and keep them in a humidified tissue culture incubator at 37 °C and 5% CO₂.

NOTE: The carryover of feeder cells may hamper the organoid differentiation. Passage the cells at least once in feeder-free conditions.

2. Passage the iPSCs at 80% confluency using enzyme-free detachment medium in ratios ranging from 1:4 to 1:12. To increase cell survival, add 10 µM Rho-associated protein kinase (ROCK) inhibitor (Y27632) after each splitting.

2. Dissociate the iPSCs (80% confluency)-Day 0.

1. Prepare Cortical Differentiation Medium I (CDMI) (**Table 1**). Prewarm CDMI medium at room temperature (22-25 °C) before adding it to the cells.
2. Wash the wells containing the iPSCs with phosphate-buffered saline (PBS) to remove dead cells and debris.
3. Add 500 µL of prewarmed Reagent A (**Table of Materials**) to each well and incubate for 5 min at

37 °C. Check under the microscope to ensure cell detachment.

4. Add 1 mL of iPSC medium to dilute reagent A to neutralize its activity.
5. Use a 1000 µL pipette to dissociate the cells by pipetting up and down and transfer the cell suspension to a 15 mL centrifuge tube.
6. Gently centrifuge the iPSCs at 125 x *g* for 5 min at room temperature (22-25 °C).
7. Carefully aspirate the supernatant to avoid disturbing the cell pellet.
8. Resuspend the pellet with 1 mL of CDMI to obtain a single-cell suspension, and count the cell number.
9. Prepare the seeding medium with 9,000 iPSCs per 100 µL in CDMI supplemented with 20 µM ROCK inhibitor, 3 µM WNT-catenin inhibitor (IWR1), and 5 µM SB431542.
10. Add 100 µL of seeding medium per well to a 96-well v-bottom plate.
11. Keep the plate in a humidified tissue culture incubator at 37 °C and 5% CO₂.

3. Neurosphere generation

1. On day 1, observe that round cell aggregates (neurospheres) with defined smooth borders are forming. Note the dead cells around the aggregates. Continue to culture in the incubator at 37 °C and 5% CO₂.
2. On day 3, agitate the plate by tapping on the sides three times to detach dead cells.

3. Add 100 μ L of CDMI supplemented with 20 μ M ROCK inhibitor, 3 μ M IWR1, and 5 μ M SB431542 to each well.
 4. Return the plate to the incubator at 37 °C and 5% CO₂.
 5. On day 6, carefully remove 80 μ L of the supernatant medium from each well. Avoid touching the bottom of the well.
 6. Add 100 μ L of CDMI supplemented with 3 μ M IWR1 and 5 μ M SB431542 to each well. Return the plate to the incubator at 37 °C and 5% CO₂.
 7. Repeat steps 5 and 6 every 3 days until day 18.
4. Transfer of neurospheres
1. On day 18, prepare Cortical Differentiation Medium II (CDMII) (**Table 1**) and add 10 mL to a 100 mm ultra-low attachment cell culture plate.
 2. Use a 200 μ L pipette with the tip cut off to transfer the round neurospheres from the 96-well plate to the 100 mm ultra-low attachment cell culture plate.
NOTE: Be gentle to avoid damaging the neurospheres by making sure the opening of the tip is wide enough and that the aggregates are not aspirated too quickly.
 3. Remove 5 mL of medium from the plate containing the neurospheres and add 5 mL of fresh CDMII.
NOTE: This procedure helps to reduce the amount of CDMI medium that may have carried over from the transfer of neurospheres.
 4. Place the plate on an orbital shaker at 70 rpm inside a humidified tissue culture incubator at 37 °C and 5% CO₂.
- NOTE:** Visually inspect the neurospheres the next day. Increase the speed of the orbital shaker if the neurospheres are clumped together or attached to the bottom of the plate.
5. Every 3 days, carefully aspirate the supernatant medium and replace it with fresh CDMII. Leave a small amount of the medium to prevent the neurospheres from drying out.
 6. On day 35, prepare Cortical Differentiation Medium III (CDMIII) (**Table 1**).
NOTE: The matrix component should be dissolved in cold CDMIII.
 7. Aspirate the medium from the plate and add 10 mL of cold CDMIII.
NOTE: It is more effective to use cold medium so that the matrix component can coat the organoids without forming clumps.
 8. After changing the medium, place the plate back on an orbital shaker at 70 rpm inside a humidified tissue culture incubator at 37 °C and 5% CO₂.
 9. Change the medium every 3-5 days depending on the rate of growth, as indicated by the color of the medium.
 10. On day 70, prepare Cortical Differentiation Medium IV (CDMIV) (**Table 1**). Use CDMIV medium until the desired age of organoids is reached. During this period, keep the plate on an orbital shaker set at 70 rpm inside a humidified tissue culture incubator (37 °C and 5% CO₂).
 11. Change the medium every 3-5 days, depending on the growth rate.

2. Immunostaining of brain organoids

1. Tissue preparation

1. Prepare 4% paraformaldehyde (PFA) solution, and place it under a safety hood.

NOTE: Wear personal safety equipment when handling PFA.

2. Collect brain organoids and gently transfer them with a blunt-tipped 3 mL plastic Pasteur pipette to a 6-well plate filled with PFA.

NOTE: Use organoids older than 40 days to allow the visualization of structures with higher cellular complexity.

3. Keep the organoids in the PFA solution for 1 h at room temperature.
4. Carefully remove the PFA with a 3 mL plastic Pasteur pipette, and wash the fixed organoids three times using PBS.
5. Store the fixed organoids at 4 °C in PBS until further use.

2. Preparation of brain organoid slices

1. Prepare a 3% agar solution and heat slowly until liquefied.
2. Place the mold (the cut end of a 10 mL syringe) on a piece of absorbent filter paper (smooth side up). Place a droplet of agar onto it.

3. Quickly take a single organoid out of the 6-well plate with a spatula and remove excessive PBS with filter paper.

NOTE: Be careful not to touch the organoid directly with filter paper.

4. Place the organoid onto the agar droplet.

5. Repeat this procedure with up to three organoids.

NOTE: Work fast to avoid solidification of the agar during this step.

6. Refill the mold with agar until all the organoids are fully covered.

7. Wait until the agar begins to solidify, and then gently transfer the entire mold containing the organoids, including the absorbent filter paper, onto a cooling element.

NOTE: If a cooling element is unavailable, store the organoids for a few minutes in a refrigerator at 4 °C.

8. In the meantime, prepare for the slicing procedure: place a razor blade (cleaned with acetone and washed with double-distilled water) into the holder of the vibratome, mount on the bath, and fill it with PBS.

9. Remove the mold from (solidified) agar and use a scalpel to trim it to form a cube.

10. Attach the agar cube containing the organoids on the carrier plate of the vibratome with adhesive gel (see the **Table of Materials**), and place it in the bath containing PBS.

11. Adjust the vibratome (see the **Table of Materials**) to cut slices at a thickness of 150 µm.

NOTE: Vibratome settings (proper angle, amplitude, frequency, and velocity of the blade) may be similar to those used for slicing fixed brain tissue derived from early postnatal animals. However, the ideal settings depend strongly on the type of the vibratome and must be determined in a first step to prevent distortion or even ripping of the tissue while cutting.

12. Start the cutting procedure. Use a glass pipette or a spatula to gently transfer each freshly cut slice into a 24-well plate filled with PBS.
 13. Store the plate containing slices at 4 °C (for up to a few days) until further processing.
 14. Transfer the slices out of the plate with a glass pipette or a spatula onto microscope slides. Use a minimum of 2 slices per slide.
 15. Carefully remove the agar and excess PBS with a syringe.
 16. Allow the slices to dry until they adhere to the slides.
NOTE: While microscope slides can be stored in plastic chambers filled with PBS at 4 °C, they should be stained as soon as possible after the slicing procedure.
3. Immunohistochemical staining
 1. Prepare the blocking solution (**Table 1**).
 2. Use a PAP pen to draw a hydrophobic border around the slices on the slide to help keep all the solutions on the slides.
 3. Carefully add the blocking solution on the slide, and incubate for 1 h at room temperature (22-25 °C). To avoid destroying the tissue, do not add the solution directly on top of the slices.
 4. Aspirate the blocking solution and apply the desired primary antibody diluted in the blocking solution.
 5. Incubate the slide overnight in a humidified chamber at 4 °C.
 6. Rinse the slide three times with 1x PBS for 10 min each.
 7. Incubate the slices with the specific secondary antibody diluted in the blocking solution and perform Hoechst staining (1:2,500) for 1 h at room temperature in the dark.
NOTE: Remember to perform negative controls to confirm there are no non-specific binding or auto-fluorescence.
 8. Rinse three times with 1x PBS for 10 min each in the dark.
 9. Add one drop of mounting medium to the slice, place a coverslip on the edge of the drop, and slowly lay the coverslip down toward the slice to avoid air bubbles.
 10. Allow the slide to rest overnight at room temperature. Apply nail polish on the border of the coverslip to further seal the slide. For long-term storage, store at 4 °C.
4. Documentation of staining
 1. To scan large images for stitching, utilize a motorized upright wide-field microscope equipped with (see the **Table of Materials** for details) a high-quality objective; DAPI filter set (e.g., excitation (EX): 340-380 nm, dichroic mirror (DM): 400 nm, barrier filter (BA): 435-485 nm); fluorescein isothiocyanate filter set (e.g., EX: 465-495 nm, DM: 505 nm, BA: 515-555 nm); Alexa594 filter set (e.g., ET 575/40; T 600 LPXR; HC 623/24); digital camera; high-performance acquisition software allowing for automated stitches and stack operations.
 2. For image handling, use an image processing program capable of generating 8-bit tif-files, cropping stitches, adjusting contrast and brightness,

merging the channels (e.g., blue, green, and red), and adding scale bars.

3. To scan details, use a motorized confocal laser scanning microscope equipped with a high-quality objective, a UV laser (EX: 408 nm), an Argon laser (EX: 488 nm), a Helium-Neon laser (EX: 543), imaging software for a confocal microscope.
4. For image handling of details, use an image processing program capable of generating maximum-intensity projections of confocal z-stacks (e.g., optical sections of 0.6 μm each), generating 8-bit tif-files, adjusting contrast and brightness, merging the channels (e.g., blue, green, and red), adding scale bars.
5. Use a graphic editor to arrange the figures.

3. Bioenergetic profiling of brain organoids

1. Preparation of organoids for bioenergetic profiling

1. Prepare the papain and DNase solution following the manufacturer's protocol.
2. Transfer 3-5 organoids into a 6-well plate. Wash them two times with prewarmed PBS.
3. Add 2 mL of prewarmed activated papain solution containing DNase. Using a blade, cut the organoids into small pieces.
4. Place the plate onto an orbital shaker set at 27 rpm inside a cell culture incubator (at 37 °C, 5% CO₂), and incubate for 15-20 min.

NOTE: The time of incubation depends on the organoid stage. Early-stage organoids can be used as they are. For organoids older than 3 months, it is recommended to cut the organoids into 2-3 pieces

before dissociation and incubate the pieces at a rocking speed set at 27 rpm for 15-20 min at 37 °C. This procedure can help remove necrotic tissue that may be present in the later-stage organoids.

5. Collect the digested tissues into a 15 mL tube and add 5 mL of organoid culture medium CDMIV (**Table 1**).
 6. Triturate the tissue with a 10 mL plastic pipette by pipetting up and down 10-15 times. Let the undissociated tissue settle down to the bottom of the tube.
 7. Carefully transfer the cell suspension to a 15 mL tube, avoiding any pieces of undissociated tissue. Filter the solution through a 40 μm cell strainer (e.g., polystyrene round-bottom tubes with cell strainer caps).
 8. Pellet the cells by centrifuging at 300 x *g* for 5 min at room temperature.
 9. Assess the cell number and quality using trypan blue.
 10. Plate the desired number (~20,000/well) of cells onto coated 96-well microplates. Change the medium 6-8 h after plating to Neuronal Medium (**Table 1**).
 11. Incubate the coated 96-well microplate in a CO₂ incubator (37 °C, 5% CO₂) for 4 days.
- #### 2. Bioenergetic profiling
1. On day 3 after replating the dissociated cells, add 200 μL of calibration solution into each well of the bottom part of the 96-well microplate, and place the top green sensor cartridge onto the hydrated microplate.

NOTE: Place the sensor cartridge on top of the microplate in the correct orientation, and ensure that the calibrant solution covers all the sensors.

2. Incubate the hydrated 96-well microplate in a non-CO₂ incubator at 37 °C overnight.
3. Turn on the analyzer to allow the instrument to stabilize at 37 °C overnight.
4. On day 4 after replating, inspect the disassociated organoid culture on the 96-well microplate under the microscope to ensure that the cells appear as a confluent monolayer.
5. Prepare Assay Medium (**Table 1**).
6. Remove Neuronal Medium from all wells with a pipette without touching the bottom of the well to prevent cell damage. Alternatively, carefully invert the whole plate and then dry it on clean paper. Work quickly to avoid cell death.
7. Wash the cells twice with prewarmed 200 µL of Assay Medium. Add Assay Medium to a final volume of 180 µL per well. Incubate the 96-well microplate in a non-CO₂ incubator at 37 °C for 1 h.
8. Prepare 10 µM solutions of mitochondrial inhibitors in Assay medium. Note that the final concentration after injection is 1 µM.
9. Load the sensor cartridge placed in the hydrated microplate with 10x solutions of the mitochondrial inhibitors.
 1. Add 18 µL of mitochondrial inhibitor 1 into port A.
 2. Add 19.8 µL of mitochondrial inhibitor 2 into port B.
 3. Add 21.6 µL of mitochondrial inhibitor 2 into port C.
 4. Add 23.4 µL of mitochondrial inhibitor 3 into port D.
10. Place the loaded cartridge in the hydrated microplate in a non-CO₂ incubator at 37 °C until the start of the assay.
11. Set up a running protocol in the instrument's software (**Table 2**).
12. Press **START**. Take the loaded cartridge from the non-CO₂ incubator and place it into the analyzer for calibration.

NOTE: Make sure the plate is inserted in the correct orientation and without the lid.
13. Once the calibration step ends, remove the calibration plate. Take the 96-well microplate from the non-CO₂ incubator and place it into the analyzer. Click on **CONTINUE** to start the measurements.
14. When the run is finished, remove the 96-well cell culture microplate from the analyzer and collect the medium from all the wells without disturbing the cells.

NOTE: The medium can be stored at -20 °C and used later for measuring the amount of lactate released by the cells in the medium using an appropriate lactate assay kit.
15. Wash the cells with 200 µL of 1x PBS in each well.
16. After removing the PBS, freeze the plate at -20 °C.

NOTE: The frozen plate can be used to quantify cells, proteins, or DNA in each well of the microplate. This quantification will be needed for normalizing the obtained bioenergetic rates. Follow

the manufacturer's instructions for cell, protein, or DNA quantification assays.

Representative Results

The protocol described here facilitates the robust generation of round organoids (**Figure 1A**). The generated organoids contain mature neurons that can be visualized using protein markers specific for axons (SMI312) and dendrites (microtubule-associated protein 2 (MAP2)) (**Figure 1B**). Mature organoids contain not only neuronal cells (MAP2-positive) but also glial cells (e.g., positive for the astrocyte marker S100 calcium-binding protein B (S100 β)) (**Figure 1B**).

By analyzing sliced brain organoids using confocal microscopy, it is possible to identify and monitor the detailed distribution and organization of different cell types and cellular structures. This could provide new insight into how mitochondrial diseases might affect nervous system development. For example, it is possible to monitor neuronal axons (SMI312-positive) and dendrites (MAP2-positive) (**Figure 2A**) or the mutual occurrence of neuronal cells (MAP2-positive) and glial cells (S100 β -positive) (**Figure 2A**). Confocal images may also help to investigate in more detail the distribution and organization of neural progenitors ((sex determining region Y) box-2 (SOX2)-positive) with respect to neurons (beta-III tubulin (TUJ1)-positive) (**Figure 2B**). Finally, brain organoids can be stained for mitochondria-specific markers (such as the outer mitochondrial membrane protein, translocase of outer membrane 20 kDa subunit (TOM20)) (**Figure 2C**).

The described protocol enables researchers to perform bioenergetic profiling of brain organoids. Using this procedure, it is possible to measure both mitochondrial metabolism using the oxygen consumption rate (OCR) (**Figure 2D**) and the glycolytic metabolism using the extracellular acidification rate (ECAR) (**Figure 2E**). Bioenergetic profiling allows monitoring how cells may modify their OCR and ECAR profiles in response to a sequential administration of mitochondrial inhibitors.

First, the ATP synthase inhibitor, oligomycin, can be applied. Oligomycin causes a drop in the OCR profile (**Figure 2D**), and therefore, identifies the OCR needed for ATP production. Upon oligomycin treatment, there may also be a compensatory increase in ECAR (**Figure 2E**), suggesting that the cells can upregulate glycolysis to prevent the metabolic stress caused by the reduction in mitochondrial metabolism. The subsequent double application of the proton ionophore, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), causes the loss of the mitochondrial membrane potential. As the oxygen molecules are now free to move, this causes a rapid increase in OCR (**Figure 2D**).

These changes in the OCR profile identify the maximal respiration capacity of the cells. The final administration of rotenone plus antimycin A causes a block of the electron transport, and therefore, a steep decrease in OCR (**Figure 2D**). ECAR may show fluctuation after treatment with FCCP and rotenone plus antimycin A (**Figure 2E**), depending on the residual glycolytic capacity of the cells. The OCR and ECAR profiles may be dramatically altered in brain organoids derived from mitochondrial patients.

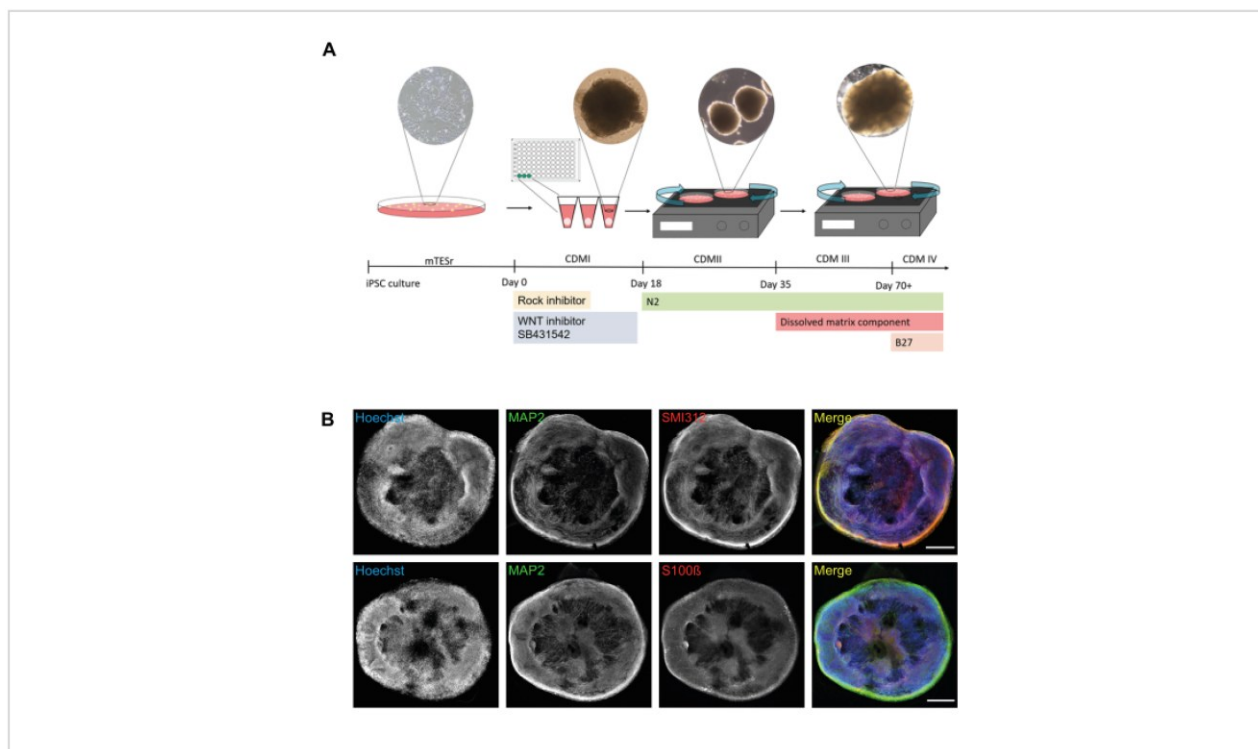


Figure 1: Generation of brain organoids from human iPSCs. (A) Schematic representation of the protocol used to produce brain organoids with corresponding transmission images. Day 0 corresponds to the dissociation of iPSCs and seeding in a 96-well plate with V-bottom using CDM I supplemented with a ROCK inhibitor, a WNT inhibitor, and SB431542. At day 18, neurospheres are transferred from the 96-well plates to 100 mm cell culture dishes with CDM II supplemented with N2. From this point onwards, the cultures are positioned on an orbital shaker. At day 35, the medium is switched from CDM II to CDM III, which also contains a dissolved matrix component (**Table 1**). From day 70 onwards, CDM III is switched to CDM IV supplemented with B27. A representative neurosphere image was taken at day 12 using a microscope camera with 10x magnification. An early organoid image was taken at day 22 using a microscope camera at 4x magnification. A mature organoid image was taken at day 40 using a microscope camera with 4x magnification. **(B)** The overall structure and cellular organization of brain organoids can be visualized using wide-field microscopy. Representative stitched wide-field images are shown to visualize the relationships between dendrites (MAP2-positive) and axons (SMI312-positive), and between neuronal cells (MAP2-positive) and presumed astrocytes (S100β-positive). The cells were counterstained with Hoechst to reveal the nuclei. All images were taken using 78 day-old brain organoids. The right column shows the overlay of three channels (merge). Scale bars = 500 μm. Abbreviations: iPSC = induced pluripotent stem cell; CDM = Cortical Differentiation Medium;



ROCK = Rho kinase; MAP2 = microtubule-associated protein 2; S100 β = S100 calcium-binding protein B. [Please click here to view a larger version of this figure.](#)

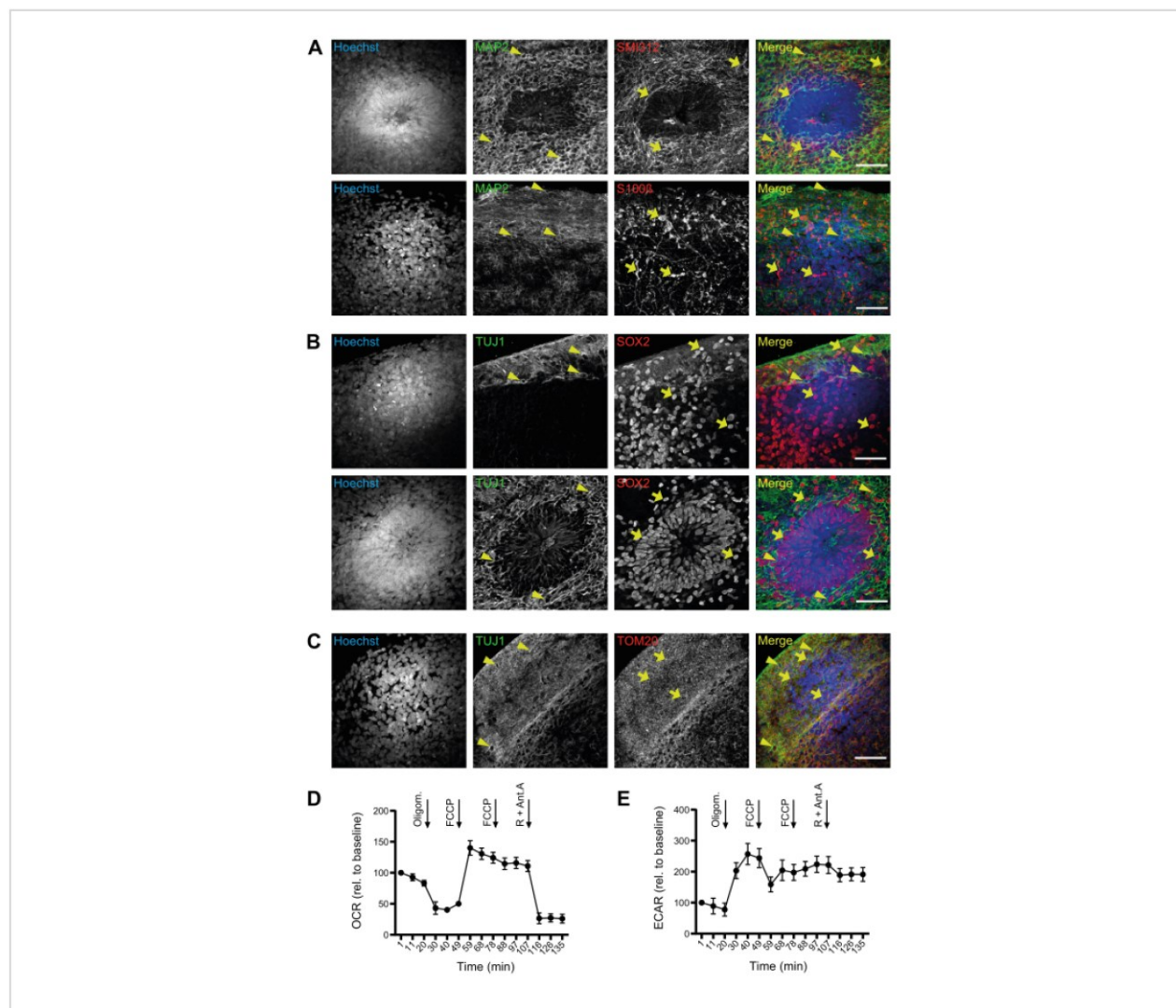


Figure 2: Visualization and bioenergetic profiling of brain organoids for mitochondrial disease modeling. The detailed organization and architecture of organoids can be analyzed using confocal microscopy. All images were taken using 78 day-old brain organoids and counterstained with Hoechst to reveal the nuclei. The right column shows the overlay of three channels (merge). Scale bars = 50 μ m. **(A)** Representative extended-focus projections (44–48 optical planes, 0.6 μ m each) addressing the interplay between dendrites (MAP2-positive, arrowheads) and axons (SMI32-positive, arrows), and between neuronal cells (MAP2-positive, arrowheads) and presumed astrocytes (S100 β -positive, arrows). **(B)** Representative extended-focus projections (14–31 optical planes, 0.6 μ m each) showing the distribution of neurons (TUJ1-positive, arrowheads) with respect to neural progenitors (SOX2-positive, arrows). **(C)** Representative extended-focus projections (20 optical planes, 0.6 μ m each) showing the distribution within neurons (TUJ1-positive, arrowheads)



of mitochondria (visualized using antibodies against the outer mitochondrial membrane protein TOM20, arrows). **(D)** Mitochondrial respiration of brain organoids can be monitored based on the profile of the OCR after sequential administration of different mitochondrial inhibitors (see text for details). **(E)** Glycolytic activity of brain organoids can be monitored based on the ECAR upon sequential administration of mitochondrial inhibitors (see text for details). For bioenergetic profiling, approximately 10-15 brain organoids were dissociated to obtain enough cells for replating onto the 96-well microplate. The bars indicate the SEMs based on the results obtained in two independent experiments. Abbreviations: MAP2 = microtubule-associated protein 2; S100 β = S100 calcium-binding protein B; TUJ1 = beta-III tubulin; SOX2 = (sex determining region Y) box-2; TOM20 = translocase of outer membrane 20 kDa subunit; OCR = oxygen consumption rate; ECAR = extracellular acidification rate; Oligom. = oligomycin; FCCP = carbonyl cyanide-p-trifluoromethoxyphenylhydrazon; R = rotenone; AntA = Antimycin A; SEMs = standard error of means. [Please click here to view a larger version of this figure.](#)

Media Composition			
CDMI (Day 0-18)			Final conc.
Glasgow-MEM	Gibco	11710-035	[1:1]
Knockout Serum Replacement (KSR)	Gibco	10828010	20%
MEM-NEAA (MEM non-essential amino acid solution)	Gibco	11140-050	0.1 mM
Sodium Pyruvate	Gibco	11360070	1 mM
2-mercaptethanol	Gibco	31350-010	0.1 mM
Penicillin and streptomycin	Gibco	15140-122	100 U/mL and 100 µg/mL
CDMII (Day 18-35)			Final conc.
DMEM/F12	Gibco	31330038	[1:1]
Glutamax	Gibco	35050-061	2 mM
N-2 Supplement (100x)	Gibco	17502-048	1%
Chemically Defined Lipid Concentrate	Gibco	11905031	1%
Penicillin and streptomycin	Gibco	15140-122	100 U/mL and 100 µg/mL
CDMIII (Day 35-70)			Final conc.
DMEM/F12	Gibco	31330038	[1:1]
Glutamax	Gibco	35050-061	2 mM
N-2 Supplement (100x)	Gibco	17502-048	1%
Chemically Defined Lipid Concentrate	Gibco	11905031	1%
Penicillin and streptomycin	Gibco	15140-122	100 U/mL and 100 µg/mL
Fetal Bovine Serum (FBS)	Gibco	10270-106	10%
Heparin	Merck	H3149-25KU	5 µg/mL
Matrigel	Corning	356231	1%
CDMIV(Day 70+)			Final conc.



DMEM/F12	Gibco	31330038	[1:1]
Glutamax	Gibco	35050-061	2 mM
N-2 Supplement (100x)	Gibco	17502-048	1%
Chemically Defined Lipid Concentrate	Gibco	11905031	1%
Penicillin and streptomycin	Gibco	15140-122	100 U/mL and 100 µg/mL
Fetal Bovine Serum (FBS)	Gibco	10270-106	10%
Heparin	Merck	H3149-25KU	5 µg/mL
Matrigel	Corning	356231	2%
B-27 supplement with Vitamin A 50x	Gibco	17504044	2%
Neuronal Medium			Final conc.
DMEM/F12	Gibco	31330038	[1:1]
N-2 supplement	Gibco	17502048	[1x]
B-27 supplement with vitamin A 50x	Gibco	17504044	[1x]
L-Ascorbic acid	Sigma Aldrich	A92902	[200 µM]
db-cAMP (dibutyl cyclic adenosine monophosphate)	Sigma Aldrich	D0627	500 µM
BDNF (brain-derived neurotrophic factor)	MACS Miltenyi	130-093-811	[10 ng/mL]
GDNF (glial cell line-derived neurotrophic factor)	MACS Miltenyi	130-096-290	[10 ng/mL]
Human TGF-β3 (transforming growth factor-beta3)	MACS Miltenyi	130-094-007	[1 ng/mL]
Assay Medium			Final conc.
Seahorse XF DMEM medium	Seahorse Bioscience	103680-100	500 mL
Sodium Pyruvate	Gibco	11360070	1 mM
L-Glutamine	Lonza	BEBP17-605E	2 mM

Glucose	Sigma Aldrich	50-99-7	10 mM
Blocking Solution			Final conc.
PBS-Tween			[1:1] 0.1% Tween
Donkey Serum	Sigma Aldrich	D9663	10%
Triton-X	Merck	X100-5ML	1%

Table 1: Details of media and solutions used for organoid generation.

Initialization	Baseline (X3)	Oligomycin Injection (X3)	FCCP Injection (X3)	FCCP Injection (X3)	Anti A + Rot Injection (X3)
Calibrate	Mix	Mix	Mix	Mix	Mix
	(04:00)	(04:00)	(04:00)	(04:00)	(04:00)
Equilibrate (12:00)	Wait	Wait	Wait	Wait	Wait
	(02:00)	(02:00)	(02:00)	(02:00)	(02:00)
	Measure (03:00)	Measure (03:00)	Measure (03:00)	Measure	Measure (03:00)
				(03:00)	

Table 2: Protocol setup for bioenergetic profiling. Description of the steps and their length in minutes using the Seahorse Wave Desktop software. Abbreviations: FCCP = carbonyl cyanide-p-trifluoromethoxyphenylhydrazon; Rot = rotenone; Anti A = Antimycin A.

Discussion

This paper describes the reproducible generation of human iPSC-derived brain organoids and their use for mitochondrial disease modeling. The protocol described here is modified based on a previously published work²⁰. One major advantage of the present protocol is that it does not require the manual embedding of each organoid into a scaffolding matrix. In fact, the matrix solution is simply dissolved into the cell culture medium. Moreover, there is no need to employ expensive bioreactors, as organoids can be cultured in standard tissue culture 6-well plates positioned onto an orbital

shaker inside the incubator. This procedure also enables the parallel cultivation of several plates containing different organoids derived from various individual lines, thereby increasing the throughput of the experiments and allowing the monitoring of potential differences emerging in the growth profiles of different organoids. We tested this protocol using different iPSCs derived from healthy controls and individuals affected by mitochondrial diseases, with consistent results.

For mitochondrial disease modeling, it is essential to use different markers to visualize the morphology and organization of the mitochondrial network. This procedure



enables the investigation of whether mitochondrial number, morphology, or distribution might be altered in brain organoids derived from patients with mitochondrial diseases. The presence and organization of neural progenitors within the brain organoids could be of crucial importance for modeling mitochondrial disorders. We recently discovered that mutations causing the mitochondrial disease, Leigh syndrome, disrupt the cellular architecture and distribution of neural progenitor cells within patient-derived brain organoids¹⁸.

For performing bioenergetic profiling, we have adapted a method that was previously described for assessing the bioenergetics of pluripotent stem cells²¹. A recent protocol described how to carry out bioenergetic profiling of organoids derived from mouse small intestine, human colon, and colorectal tumors²². However, those organoids are quite small compared to brain organoids, and therefore, a different protocol, such as the one reported here, is needed for brain organoids. We recently employed this protocol for assessing the bioenergetic profile of human brain organoids carrying mutations in the surfeit locus protein 1 gene (*SURF1*) that causes the severe mitochondrial disease, Leigh syndrome¹⁸. We found that the OCR profile is particularly affected in Leigh syndrome organoids, as shown by a significant decrease in the basal OCR level, the ATP production rate, and the maximal respiration rate¹⁸.

In conclusion, we present here a detailed protocol for the robust generation of human brain organoids and describe how to perform experiments that would be important for the investigation of the disease mechanisms underlying mitochondrial diseases. Human brain organoids may also be of critical importance for elucidating the mitochondrial diversity in the human brain and its role in human health

and diseases²³. It is important to clarify that brain organoids generated with currently available protocols, including the one described here, still bear limitations. These include, for example, the lack of vascularization and the absence of microglia population²⁴. These aspects need to be taken into consideration to interpret the results correctly.

For example, the lack of vasculature and microglia could limit compensatory mechanisms that may be in place *in vivo*. Patient-derived brain organoids might thus exhibit defects that are stronger than the ones observed in patients^{17, 18}. Moreover, despite a general reproducibility of this protocol²⁰, line-to-line heterogeneity can be observed. To this end, when performing disease modeling studies, it is always important to systematically quantify the uniformity of control and patient organoids by assessing the patterns of morphology (size, layer) and the distribution of molecular markers across different organoids.

Finally, it is not possible to generate brain organoids from a single iPSC, limiting the feasibility of large-scale genetic screening with CRISPR/Cas9. Given the pace of research, it is likely that some of the current limitations of the protocol described here will soon be overcome. Optimized protocols will become available. These 3D models of mitochondrial diseases will hopefully enable the eventual discovery of implementable therapies for mitochondrial diseases, which are detrimental, and for incurable diseases with highly unmet medical needs.

Disclosures

The authors declare no competing financial or non-financial interests.

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3.2. HIGH-CONTENT ANALYSIS OF NEURONAL MORPHOLOGY IN HUMAN iPSC-DERIVED NEURONS

Lickfett S, **Menacho C**, Zink A, Telugu NS, Beller M, Diecke S, Cambridge S, Prigione A.

Abstract

We present a high-content analysis (HCA) protocol for monitoring the outgrowth capacity of human neurons derived from induced pluripotent stem cells (iPSCs). We describe steps to perform HCA imaging, followed by quantifying the morphology of dendrites and axons within a high-throughput system to evaluate neurons obtained through various differentiation approaches. This protocol can be used to screen for modulators of neuronal morphogenesis or neurotoxicity. The approach can be applied to patient-derived iPSCs to identify patient-specific defects and possible therapeutic strategies.

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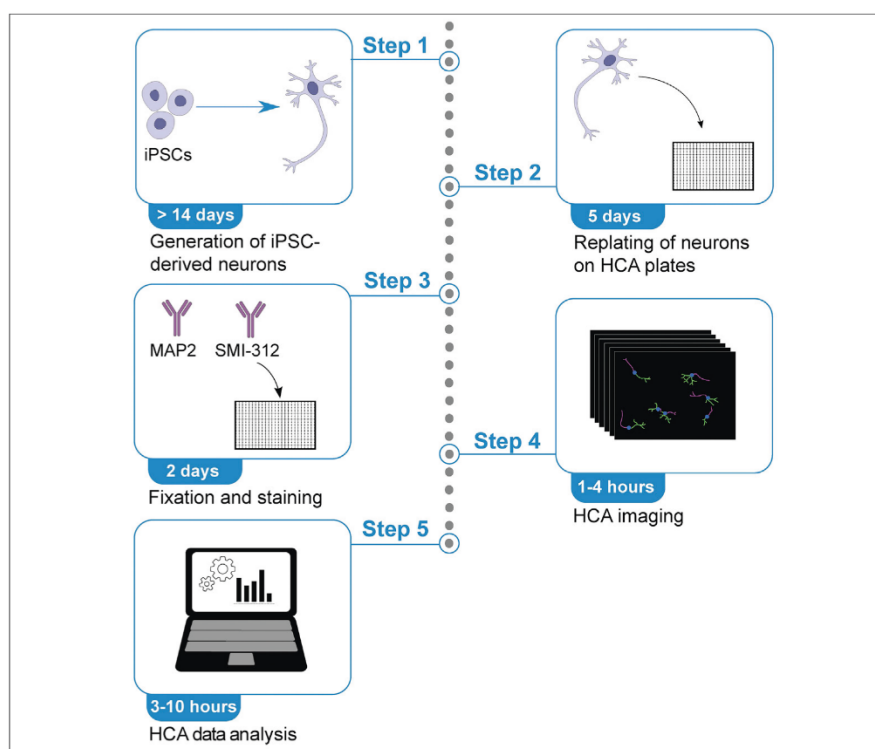
Impact Factor: 1.3 (2023)

Contribution: development and investigation of the CellProfiler analysis pipeline to measure neurite outgrowth in induced neurons derived from iPSC; manuscript edition and revision.

STAR Protocols

Protocol

High-content analysis of neuronal morphology in human iPSC-derived neurons



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Highlights

High-content analysis
of human iPSC-
derived neurons

Automated
microscopy to
quantify neuronal
morphology

Quantification of
axonal and dendritic
outgrowths

Data analysis based
on open-source
software CellProfiler

We present a high-content analysis (HCA) protocol for monitoring the outgrowth capacity of human neurons derived from induced pluripotent stem cells (iPSCs). We describe steps to perform HCA imaging, followed by quantifying the morphology of dendrites and axons within a high-throughput system to evaluate neurons obtained through various differentiation approaches. This protocol can be used to screen for modulators of neuronal morphogenesis or neurotoxicity. The approach can be applied to patient-derived iPSCs to identify patient-specific defects and possible therapeutic strategies.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Protocol

High-content analysis of neuronal morphology in human iPSC-derived neurons

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SUMMARY

We present a high-content analysis (HCA) protocol for monitoring the outgrowth capacity of human neurons derived from induced pluripotent stem cells (iPSCs). We describe steps to perform HCA imaging, followed by quantifying the morphology of dendrites and axons within a high-throughput system to evaluate neurons obtained through various differentiation approaches. This protocol can be used to screen for modulators of neuronal morphogenesis or neurotoxicity. The approach can be applied to patient-derived iPSCs to identify patient-specific defects and possible therapeutic strategies.

For complete details on the use and execution of this protocol, please refer to Zink et al. (2020) and Inak et al. (2021). The protocol can be used in combination with Zink et al. (2022).

BEFORE YOU BEGIN

The protocol described here enables to assess the morphology of human neurons derived from induced pluripotent stem cell (iPSCs). Before starting the protocol, it is important to obtain iPSC-derived neurons that can be robustly grown and visually monitored. Here, we describe specific steps for neurons differentiated from a human iPSC line engineered to express the pan-neuronal transcription factor Neurogenin 2 (NGN2) construct in the AAVS1 locus in an inducible manner. Using this iPSC line (BIHi005-A-24), it is possible to activate the expression of NGN2 upon doxycycline (DOX) exposure which converts the iPSCs into a pure neuronal population in about 14 days (Zhang et al., 2013) (Figure 1). However, the HCA protocol described here can also be reliably applied to iPSC-derived neurons obtained using other differentiation approaches. For example, we successfully used this protocol for dopaminergic neurons obtained by exposing iPSCs to specific growth factors and small molecules (Inak et al., 2021; Zink et al., 2020).

Institutional permissions

Studies with control iPSCs were performed in accordance with the approval by the Ethic Committee of the Medical Faculty of Heinrich Heine University (study number: 2020-967_2).

Preparation of cell culture materials and buffers

Required media and solutions used in this protocol can either be prepared in advance and stored as indicated, or can be made fresh on the day of the experiment. Please refer to materials and equipment for a complete list of recipes.



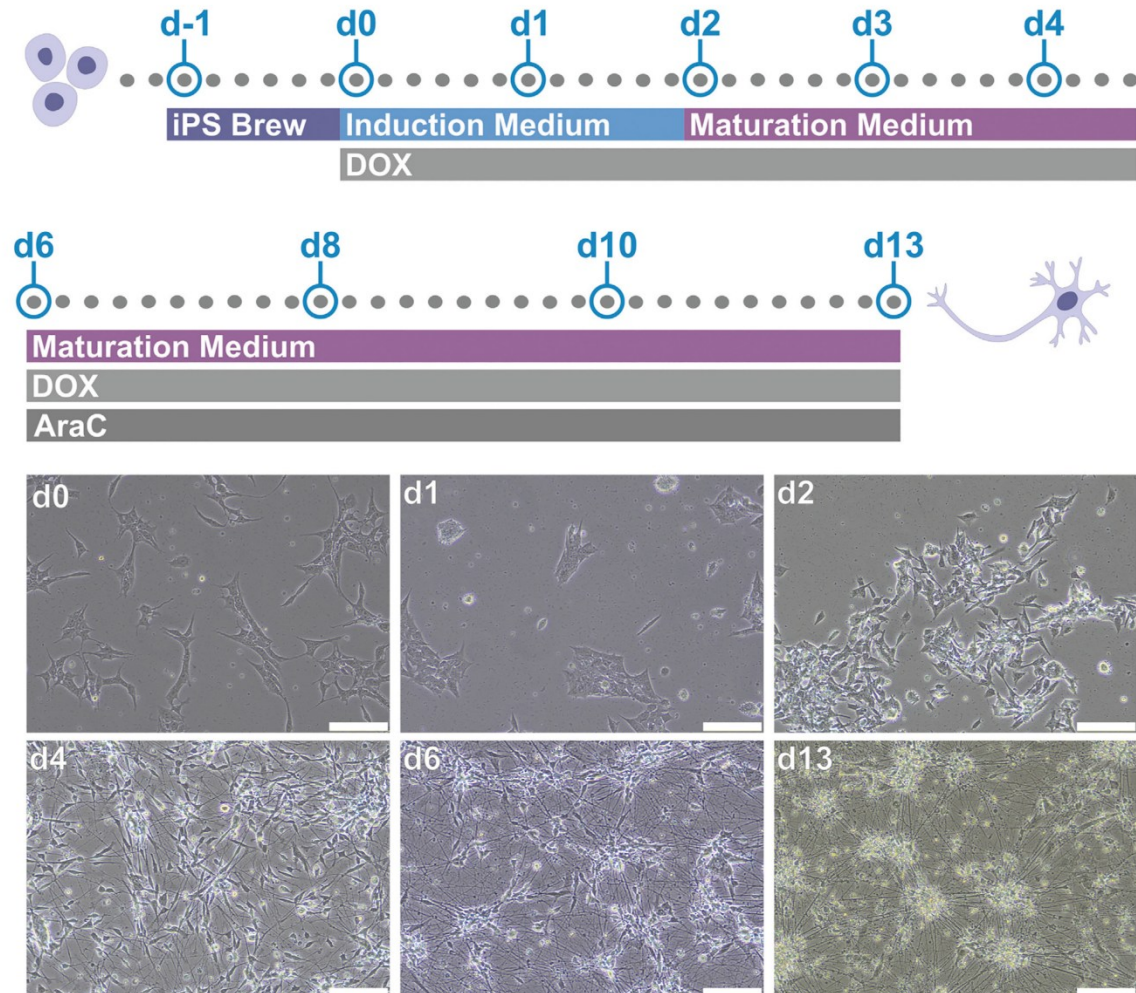


Figure 1. Neurons differentiated from human induced pluripotent stem cells (iPSCs)

Upper panel, step-by-step protocol for the NGN2 induction from the engineered iPSC line BHI005-A-24. Lower panel, exemplary images of the differentiating neurons at different time points. Scale bar: 200 μ m.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-Guinea Pig IgG (H+L), highly cross-adsorbed, CF [™] 488A antibody produced in donkey (1: 1000)	Sigma-Aldrich	Cat#SAB4600033
Anti-MAP2 polyclonal antibody (guinea pig) (1: 1000)	Synaptic Systems	Cat#188004
Anti-Neurofilament mixture of monoclonal antibodies, SMI312 (mouse) (1:500)	BioLegend	Cat#837904
Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 568 (1: 1000)	Invitrogen	Cat#A-11031

(Continued on next page)



Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
16% Paraformaldehyde (PFA)	Thermo Fisher Scientific	Cat#28906
B27	Gibco	Cat#17504044
BDNF	MACS Miltenyi	Cat#130-096-811
Bovine Serum Albumin	Sigma-Aldrich	Cat#A9647
Cytosin-1-β-D-arabinofuranosid (AraC)	Sigma-Aldrich	Cat#C1768
DMEM/F-12	Gibco	Cat#31330095
Doxycycline hydrochloride (DOX)	Sigma-Aldrich	Cat#D3072
Geltrex	Gibco	Cat#A1413201
GlutaMAX	Gibco	Cat#35050061
Hoechst 33342	Invitrogen	Cat#H3570
Laminin	Sigma-Aldrich	Cat#L2020
MycoZap	Lonza	Cat#VZA-2012
Neurobasal	Gibco	Cat#21103-049
NT-3	Biozol/PeproTech	Cat#450-03
PBS w/o calcium and magnesium	Gibco	Cat#14190144
ROCK inhibitor Y-27632	Enzy Life Sciences	Cat#ALX-270-333-M005
Sodium Azide	Sigma-Aldrich	Cat#71289
StemPro Accutase	Thermo Fisher Scientific	Cat#A1110501
Triton X-100	Bio-Rad	Cat#161-0407
Deposited data		
CellProfiler pipeline neurite outgrowth	This paper	https://doi.org/10.5281/zenodo.6642365
Experimental models: Cell lines		
Human: BHI005-A-24 cell line	Berlin Institute of Health	N/A
Software and algorithms		
CellProfiler 4.2.1.	Carpenter et al. (2006)	https://cellprofiler.org/
Other		
384 well Cell Culture Microplate, PS, F-Bottom, black TC, μCLEAR	Greiner Bio-One	Cat#781091
CytoSmart Cell Counter	Greiner Bio-One	Cat#6749
Operetta® CLS™	PerkinElmer	Cat#HH16000000

MATERIALS AND EQUIPMENT

Maturation medium for NGN2 neurons		
Reagent	Final concentration	Amount
Neurobasal medium	n/a	48.3 mL
B-27	1 ×	1 mL
GlutaMAX	1 ×	0.5 mL
BDNF (10 μg/mL)	10 ng/mL	50 μL
NT-3 (10 μg/mL)	10 ng/mL	50 μL
Laminin (10 μg/mL)	0.2 μg/mL	10 μL
MycoZap	1 ×	100 μL
DOX (3 mg/mL, add directly before use)	3 μg/mL	n/a
AraC (add only from day 6)	2 μM	25 μL
Total	n/a	50 mL

Store at 2°C–8°C for up to 2 weeks. Always add fresh DOX directly before use to the medium.

Note: Thawed doxycycline (DOX) aliquots should be kept at 2°C–8°C in the dark and used for maximal 1 week.



Alternatives: If using a different neuronal differentiation approach, other media might be necessary.

8% Paraformaldehyde (PFA)

Reagent	Final concentration	Amount
16% PFA	8%	10 mL
PBS w/o calcium and magnesium	n/a	10 mL
Total	n/a	20 mL

Store at 2°C–8°C for up to 1 week or at –20°C for several months.

⚠ CRITICAL: PFA is flammable, toxic, carcinogenic, and it is an irritant for the skin, eyes, and respiratory tract. A lab coat, face mask, and safety goggles should be worn when handling PFA. All steps should be performed inside a fume hood; waste should be disposed in special containers in accordance with applicable regional and national laws and regulations.

Blocking solution for immunofluorescence staining

Reagent	Final concentration	Amount
Bovine Serum Albumin 10% (w/v)	3%	9 mL
Triton-x-100	0.5%	150 μ L
PBS w/o calcium and magnesium	n/a	21 mL
Total	n/a	30 mL

Store at –20°C for several months.

Alternatives: Antibodies.

We use neuronal markers SMI312 to identify axons and MAP2 to identify dendrites (Hoffmann et al., 2021). However, other markers may also be used. This protocol can be adapted to screen using a general neurite staining, for example beta-III-tubulin (TUJ1).

Alternatives: High-content analysis imaging platform.

Here, we describe the use of HCA system Operetta® CLS™ (Perkin Elmer). However, we successfully applied the same HCA protocol also to the HCA system CellInsight High-Content Screening (Thermo Scientific).

STEP-BY-STEP METHOD DETAILS

Cell seeding and culturing on HCA plates

⌚ Timing: 5 days

In this step, iPSC-derived neurons are seeded onto coated HCA plates and cultured for several days to allow for neurite outgrowth (Figure 1).

1. Preparation of HCA plates.

- Coat a black wall, clear flat-bottom 384-well plate suited for imaging using 50 μ L geltrex coating solution (1:100 dilution in DMEM-F/12) per well, and incubate for 1 h at 37°C.

Note: If HCA plates are not directly used after coating, they can be wrapped with parafilm and stored for up to two weeks at 2°C–8°C.



Alternatives: Instead of geltrex, coating with matrigel is also possible. In our hands, the two coating materials give comparable results and provide good neuronal attachment and outgrowth. However, matrigel is not a defined formula, and thus it may lead to potential batch-to-batch variability. Other coating substances can also be employed, depending on the neural cell type of use.

Δ CRITICAL: It is crucial to employ plates with a bottom suitable for high resolution imaging, for example glass or μ Clear® bottom.

2. Seeding of iPSC-derived neurons (Figure 1).
 - a. For 6-well plates, split iPSC-derived neurons by applying 0.5 mL accutase® (an enzyme mixture with proteolytic and collagenolytic activity) onto the cells for 3 min at room temperature (RT, 20°C–25°C).
 - b. Dilute accutase activity by adding 1 mL of culture media and wash off the cells. Collect the cells and transfer them into a 15 mL falcon tube with 7.5 mL PBS to further dilute accutase activity. Wash the well with 1 mL of culture media and transfer any of the remaining cells into the same 15 mL falcon tube.
 - c. Centrifuge for 5 min at 100 $\times$ g, remove the supernatant, and re-suspend the cells in maturation media. Count the cells using a hemocytometer or a cell counter.
 - d. Mix the cell suspension with maturation medium containing fresh DOX and 10 μ M ROCK-inhibitor to a concentration of around 40,000 cells/mL. Pipette 50 μ L of the cell suspension per well (384-well plate).
 - e. Incubate the cells in a tissue culture incubator at 37°C and 5% CO₂.

Note: Optimal seeding density might need to be adapted depending on the specific neuronal type and iPSC line used for differentiation. Low density helps with downstream image analysis, while high density usually improves cell survival. In our hands 2,000–5,000 cells per well (40,000–100,000 cells/mL) yield the best results in this HCA pipeline.

3. Culturing of iPSC-derived neurons.
 - a. Every second day, remove half of the culture medium and replace it with maturation medium containing fresh DOX.

Δ CRITICAL: Always carefully add or aspirate medium to avoid cell detachment. In our hands, cells adhere less strongly in 384-well plates compared to wells with standard glass cover slips.

Note: If there are many dead cells, you can remove the culture medium completely and wash the cells with PBS. However, this should only be done if the cells attach strongly to the bottom surface.

Note: The optimal time point for fixation might need to be adapted depending on the specific neuronal type and iPSC line used. We recommend fixing the iPSC-derived neurons 4 days after seeding them to ensure sufficient level of neurite outgrowth for effective classification by the HCA software.

Fixation and staining of iPSC-derived neurons

⌚ Timing: 2 days

In this step, iPSC-derived neurons are fixed and immunostained for axonal and dendritic markers.

4. Fixation of iPSC-derived neurons.



- a. Add 50 μ L of the 8% PFA solution directly to the culture medium for a final 4% PFA solution. Incubate for 15 min at RT under a fume hood.
- b. Aspirate the culture medium / PFA solution and carefully wash each well three times with 50 μ L PBS. After the last washing step, leave 50 μ L of 0.05% sodium azide in PBS in each well to improve conservation, or add the blocking solution for the subsequent staining steps.
- c. If the cells will not be stained directly after fixation, seal the plate with parafilm and store it at 2°C–8°C.

Note: The fixative is added directly into the medium to avoid detachment of the cells during aspiration of the culture medium. It is possible to add 4% PFA to the culture medium (for a final PFA concentration of 2%), but then the incubation time doubles. If cells adhere well, it is also possible to aspirate the culture medium and add 4% PFA directly to the well.

▮▮ Pause point: The plate can be stored at 2°C–8°C for a few weeks before staining.

5. Staining of iPSC-derived neurons.
 - a. Aspirate the PBS and add 50 μ L of the blocking solution to each well. Incubate at RT for 1 h or overnight (12–24 h) at 2°C–8°C.
 - b. Prepare the primary antibody solution in blocking buffer.
 - i. Dilute the SMI312 antibody 1:500 and the MAP2 antibody 1:1000.
 - c. After the incubation time, aspirate the blocking solution and add 20 μ L of the primary antibody solution per well. Seal the plate with parafilm and incubate overnight (12–24 h) at 2°C–8°C.
 - d. The next day, aspirate the primary antibody solution, and wash each well three times with 50 μ L PBS.
 - e. Prepare the secondary antibody solution in blocking buffer including Hoechst 3342.
 - i. Dilute both secondary antibodies 1:1000 and Hoechst 33342 1:2500.
 - f. Add 20 μ L of the secondary antibody solution per well. Incubate in the dark at RT for at least 1 h.
 - g. After the incubation time, aspirate the secondary antibody solution and wash each well three times with 50 μ L PBS. After the last washing step, leave 80 μ L of 0.05% sodium azide in PBS in each well.
 - h. For long-term storage of the plate, seal the plate with parafilm and keep at 2°C–8°C. Check regularly that there is sufficient PBS inside the wells of the plate to prevent the fixed cells from drying out.

Note: The fluorescent dyes on secondary antibodies are light-sensitive and should not be exposed to incident light. After incubation with secondary antibodies, the plates should also always be protected from the light.

Note: The exact antibody dilutions might need to be adapted.

Note: Instead of Hoechst, other nuclear dyes can also be used (e.g., DAPI).

HCA imaging

⌚ Timing: 0.5–3 h (depending on number of wells and image acquisition settings)

In this step, using a HCA imaging platform, images of the wells are acquired in an automated fashion. The described settings are specific for the Operetta® CLS™ together with its software Harmony (Perkin Elmer). Comparable settings exist for other HCA imaging platforms as well.

6. Set up the HCA platform and microscope settings.
 - a. Place the plate in the HCA imaging platform.
 - b. Select the plate format.
 - i. Check manufacturers' comments on plate usage.



- c. Select the 20× Air, NA 0.4 objective.
- d. Choose the non-confocal operational mode.
- e. Choose a binning of '1'.
- f. Define the plate layout.
 - i. Select the wells and the fields within each well that should be imaged.

Note: These are the settings we determined to work most effectively for us. However, each of the settings might need to be adapted to your specific neuronal cell type, iPSC line, or HCA platform.

Note: It is possible to use confocal operation mode instead of non-confocal. Confocal imaging can provide higher resolution especially if cells adhere not as a monolayer but partially stack upon each other in multiple layers. However, confocal imaging requires higher exposure time and LED power, thus leading to prolonged acquisition time.

Note: A higher binning is also possible, which increases signal-to-noise ratio, shortens exposure time and thus reduces acquisition time. However, this can lead to decreased spatial resolution.

Note: Determine which and how many fields need to be imaged to represent the well. In our experience, neurons are likely to attach more closely to the border of the well than in the middle. Therefore, it is useful to include outer fields in the imaging set-up. It is important to point out that outer fields include the border of the well. Thus, individual field comparisons should be avoided, as it is difficult to compare fields with borders (i.e., outer fields) to fields without borders. We recommend to always perform statistical tests on all fields of each well combined.

- g. Create a channel for each fluorophore you are using and determine the exposure time, LED power and height (z-off) for each channel.
 - i. Select one well and field of a well and click snapshot.
 - ii. On the image control panel, use the histogram to verify the maximum value. We recommend decreasing exposure time or LED power to keep the maximum value below 60,000.
 - iii. To determine the height, acquire a z-stack (click 'Test', not 'Snapshot') and identify the image with the sharpest focus. Enter the respective height for each channel.

Note: You can set a different height for each channel. This can be helpful as neurites sometimes tend to be at a different focus plane than the nuclei.

- 7. HCA Imaging.
 - a. Go to 'Run Experiment', name it and click 'Start'.
 - b. After the run is finished, export the data as .tiff files.

Note: When considering the settings, it is important to find a balance between high-quality images and the time needed to acquire them. As an example, image acquisition with 3 channels of one plate with 100 wells selected and 13 fields of view per well takes about 25 min. With the 20 × objective, it is possible to acquire up to 25 fields of view. We recommend to capture at least 12 fields of view per image to allow robust data collection. However, before starting the acquisition, we suggest imaging some wells with all 25 fields in order to examine whether the cells grow uniformly throughout the well.

Note: If cells are not in focus, include additional Z-planes. This however increases the acquisition time.

Note: If working with neurons derived from patients or exposed to treatments, always set up the image acquisition based on a well containing control/untreated neurons. But before



starting the analysis, remember to also image the wells containing the diseased/treated neurons. This is important to avoid out-of-focus images and inadequate exposure times.

HCA image analysis

⌚ Timing: 3–10 h (depending on the computer capacity and number of images to be processed)

This step describes the HCA image analysis for the quantification of neurite outgrowth using the open-source software CellProfiler, which allows the processing of images from high-throughput experiments (Figures 2A and 2B, and Methods video S1).

The HCA pipeline, example images to test the pipeline and a video of all the steps can be downloaded here: <https://github.com/StemCellMetab/neurite-outgrowth>.

8. Setting up the CellProfiler analysis pipeline.
 - a. Open the CellProfiler analysis pipeline.
 - b. Go to Images.
 - i. Add images by dragging and dropping them into CellProfiler.
 - c. Go to Metadata. Enter the extraction method and metadata source.
 - i. To set an expression to enable CellProfiler to interpret metadata from a file name or folder, click on the magnifying glass. The test text should reflect the name correctly. In case it does not, click 'Guess' to update the text or edit it manually.
 - ii. Click 'Submit'.
 - iii. Click 'Update' to extract and display the metadata in the CellProfiler window.
 - d. Go to NameAndTypes and adjust the settings according to your staining and the naming of your images.
 - i. In the default settings of our pipeline, channel 1 is set as Hoechst (staining of nuclei), channel 2 is set as MAP2 (dendritic staining), and channel 3 is set as SMI312 (axonal staining).
 - e. Set the default input and output folders.
 - i. Select the 'File' dropdown menu and select 'preferences'. Select 'Browse' next to the 'Default Output Folder' and 'Default Input Folder'.
 - ii. Enter a filename for saving.
 - f. Go to the ExportToSpreadsheet Module at the end of the pipeline.
 - i. Enter a name at 'filename prefix'.

Note: If you have a very large dataset, CellProfiler might not be able to export to a spreadsheet. In that case, replace the 'ExportToSpreadsheet' module with the 'ExportToDatabase' module and adjust the settings to your needs.

9. Testing and adjusting the pipeline.
 - a. Click on 'Start Test Mode'.
 - b. A green check-mark will appear next to the modules that contain no errors. A red 'x' will appear next to the modules that do contain errors. The 'ExportToSpreadsheet' module always has a yellow triangle during the test mode. If you hover with the mouse over the red 'x', CellProfiler will spell out the exact error.
 - c. Use the 'step' button to go through each module. A short description is given in Table 1. Visually check the images and adjust each module according to your cells and image intensity (see also Figure 2B).
 - d. Test the settings on multiple image sets. Select the 'Test' dropdown menu to select specific image sets to adjust the settings for images from controls as well as treated cells.
10. Starting the analysis.
 - a. Exit the test mode (click 'Exit Test Mode'), disable all windows and click the 'Analyze Images' button.

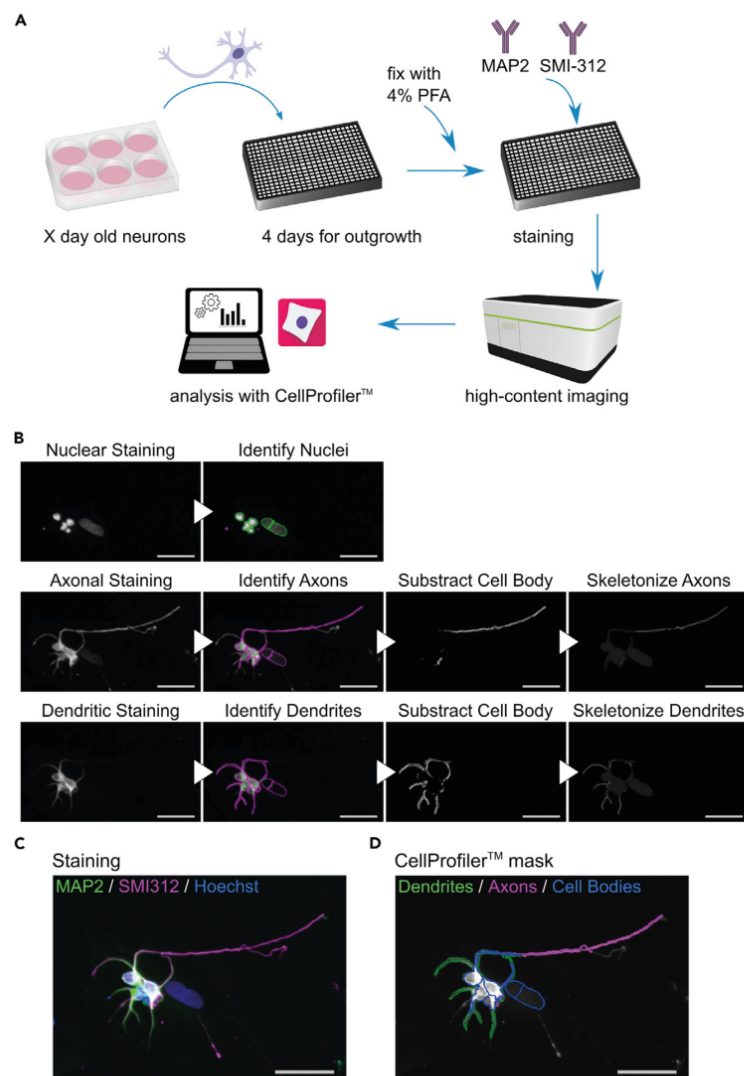


Figure 2. HCA pipeline for neurite quantification

(A) Schematic representation of the individual steps of the HCA protocol with iPSC-derived neurons.

(B) Stepwise exemplar images of the HCA protocol generated by CellProfiler analysis. Scale bar: 50 μ m.

(C) Merged color image obtained by HCA microscopy. Scale bar: 50 μ m.

(D) Image showing the CellProfiler mask superimposed over the merged grayscale image obtained by HCA microscopy. Scale bar: 50 μ m.

- b. CellProfiler will now analyze the image sets and export the results to a spreadsheet on the selected drive and folder. The pipeline will also save a spreadsheet containing the used settings (*filename prefixExperiment*).

Note: MAP2 and SMI-312 staining distinguish between dendrites and axons, respectively (Figures 2C and 2D). The HCA protocol can be adapted also to capture neurites in general,

Table 1. Description of each module of the CellProfiler pipeline

Module	Description
IdentifyPrimaryObjects	Identifies the nuclei via Hoechst staining. Determine the typical diameter empirically by testing different image sets. Nuclei touching the border of the image are removed.
Threshold	Converts the SMI312 grayscale image into a binary image for masking the nuclei in the next step.
MaskObjects	Keeps only those nuclei with at least 20% overlap with the SMI312 staining. This way only neuronal nuclei will remain. This might be necessary for primary cultures with a mixed assortment of cell types.
IdentifySecondaryObjects	Identifies axons (+cell body) in the SMI312 image around the previously identified and masked nuclei.
IdentifySecondaryObjects	Identify dendrites (+cell body) by using the MAP2 image around the previously identified and masked nuclei.
MaskObjects	Both SMI312 and MAP2 stain the cell body next to axons and dendrites, respectively. By masking the SMI312 staining with the MAP2 staining the overlap is considered the cell body.
IdentifyTertiaryObjects	Removes the cell body from the 'axons+cell body' identified before so that only the axons will remain.
IdentifyTertiaryObjects	Removes the cell body from the 'dendrites+cell body' identified before so that only the dendrites will remain.
MeasureObjectSizeShape	Measures the size and shape of axons and dendrites.
ConvertObjectsToImage	The skeleton module requires a binary picture, so this module converts the identified axons to a binary image.
ConvertObjectsToImage	The skeleton module requires a binary picture, so this module converts the identified dendrites to a binary image.
MeasureObjectSkeleton	Measures the skeletal features (branches, endpoints, trunks) for the axons starting from the cell body.
MeasureObjectSkeleton	Measures the skeletal features (branches, endpoints, trunks) for the dendrites starting from the cell body.
ExportToSpreadsheet	Exports the data (number of objects, measurements, etc.).

which can be visualized using an antibody against beta-III-tubulin (TUJ1). In this case, change 'NamesAndTypes' accordingly, and delete the modules that are duplicated.

Note: Further statistical analysis of the data and graphical representations might be necessary, and can be carried out using different software (e.g., Microsoft Excel or GraphPad Prism).

EXPECTED OUTCOMES

The protocol described here enables monitoring neuronal morphology and quantifying outgrowth capacity of human neurons derived from iPSCs using high-content analysis (HCA). Upon re-plating onto a 384-well-plate, iPSC-derived neurons should attach and form neurites within 1–5 days. This HCA analysis pipeline allows quantification of both axonal and dendritic length, number of branch points, staining signal areas, and other parameters (Figure 2). Here, we applied the HCA protocol to human neurons generated through the over-expression of NGN2 (Zhang et al., 2013) using engineered iPSCs. Our protocol can be employed to address the impact on neurite outgrowth and branching of iPSC-derived neurons exposed to different drugs, siRNAs, or guide RNAs. The HCA pipeline can also be applied to neurons differentiated from patient-derived iPSCs to possibly unveil disease-specific neuronal defects (Inak et al., 2021). In combination with the assessment of live mitochondrial activity (Zink et al., 2022), this protocol can also enable the identification of modulators of human neuronal and mitochondrial toxicity (Zink et al., 2020).

LIMITATIONS

This protocol does not allow to determine the functional properties of iPSC-derived neurons. To assess these properties, calcium imaging or electrophysiological measurement should be carried out. In order to investigate functional neuronal aspects, co-culture with glia cells might also be required, as pure neurons alone may not develop functional synapses (Klapper et al., 2019).

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With this protocol, it is also not possible to perform Scholl analysis, which is a widely used approach for assessing dendritic arborization. In fact, even though a Scholl analysis plugin for CellProfiler is available, its functions appear quite limited, and so far we were not able to implement it into our HCA pipeline. One specific challenge is that Scholl analysis requires neurons to be imaged as individual cells. However, this is not easy to achieve for human iPSC-derived neurons that tend to grow in dense cultures to maintain their survival. Perhaps, one can envision that the use of sparsely fluorescently labeled cultures, for example obtained through low-titer virus transfection using a reporter gene such as GFP, might limit the neuronal signals that need to be resolved, thereby allowing to visualize single neurons for Scholl analysis. We hope that these issues can be resolved soon to enable the inclusion of Scholl analyses in future HCA outgrowth pipelines of human iPSC-derived neurons.

Another limitation of our protocol is that, given the complex morphology of neurons, axonal/dendritic crossing-overs from two or more neighboring neurons may take place. This phenomenon likely adds 'noise' to the estimation of both lengths and ramifications. By using overlapping SMI312 and MAP2 staining to determine the cell body, neurite crossing and overlap of neighboring cells might falsely be labeled as cell body and thus be excluded from the neurite analysis. This is a problem inherent to automated imaging approaches of cultured neurons. In our opinion, it is therefore essential to first optimize the assay to determine the best seeding density and fixation time point for each neuronal cell type. Careful, manual evaluation of the images is key, and experimenters should not rely solely on automated processing and quantification.

TROUBLESHOOTING

Problem 1

Neurons are too confluent or too sparse to be properly visualized and analyzed (see [cell seeding and culturing on HCA plates](#), step 2).

Potential solution

Determine the optimal cell density in a pilot experiment. Neuronal seeding should be sufficiently dense to ensure survival, but also sparse enough to allow proper imaging and downstream quantification analysis.

Problem 2

The staining does not highlight distinct neurite structures (see [cell seeding and culturing on HCA plates](#), step 3).

Potential solution

In case neuritic structures are not yet fully formed, they may exhibit lower staining intensities because they do not possess a mature protein composition. This problem could be overcome by fixing the differentiating neurons at later time points or by using higher antibody concentrations. We recommend to test multiple time points in pilot experiments, and check that there is sufficient neurite outgrowth. At the same time, the neurite network should not be too dense, as this will compromise the efficient detection in image analyses. In our hands, allowing iPSC-derived neurons to grow for 4 days after splitting on the HCA plate is sufficient to ensure proper neurite outgrowth. However, the optimal fixation time point might need to be determined for different neuronal types. In our experience, iPSC-derived neurons grown for up to 7 days can still be readily assessed by this HCA pipeline. At longer time points, the complex structure of neurons may prevent obtaining reliable HCA results.

Problem 3

Neurons detach after washing steps or after fixation (see [fixation and staining of iPSC-derived neurons](#)).



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Potential solution

- Try different coatings or increase the concentration of the coating material.
- Reduce the washing steps.
- Seed a higher number of cells to compensate for cell loss during fixation and staining.

Problem 4

Cells are out-of-focus (see [HCA imaging](#), step 6, g).

Potential solution

- Try confocal mode or different Z-stacks.
- Optimize the seeding conditions. Ideally, cells should grow as individual cells.
- Check if the plate used is suitable for HCA imaging and whether the optical transparency is sufficient. We suggest to check the web page of the manufacturer of the HCA instrument to identify recommended plates suitable for imaging on that instrument.

Problem 5

The CellProfiler pipeline does not generate results (see [HCA image analysis](#)).

Potential solution

- Try to determine the point of the pipeline where an error may have occurred. CellProfiler shows a red 'x' if there is a problem within one module.
- Check that you changed 'NamesAndTypes' according to the naming system of your images.
- Check if the default input and output folders are set correctly.
- Start 'Test Mode' and check on multiple image sets that all settings (size, threshold, etc.) match your images.
- For other more specific errors, check CellProfiler's forum, where many issues are discussed and covered.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Alessandro Prigione (alessandro.prigione@hhu.de).

Materials availability

This study did not generate new unique materials.

Data and code availability

The CellProfiler pipeline for neurite outgrowth generated during this study is available at Github: <https://github.com/StemCellMetab/neurite-outgrowth> and has been deposited at Zenodo: <https://doi.org/10.5281/zenodo.6642365>.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.xpro.2022.101567>.

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AUTHOR CONTRIBUTIONS

Conceptualization, S.L., A.Z., and A.P.; methodology, S.L., A.Z., M.B., A.P., S.C., and C.M.; investigation, S.L., N.S.T., S.D., and C.M.; writing – original draft, S.L.; writing – review and editing, S.L., A.P., and S.C.; funding acquisition, A.P., S.C., and M.B.; resources, M.B., N.S.T., and S.D.; supervision, A.P. and S.C.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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3.3. MUTANT HUNTINGTIN IMPAIRS NEURODEVELOPMENT IN HUMAN BRAIN ORGANIDS THROUGH CHCHD2- MEDIATED NEUROMETABOLIC FAILURE

Lisowski P*, Lickfett S*, Rybak-Wolf A, **Menacho C**, Le S, Pentimalli TM, Notopoulo S, Dykstra W, Oehler D, Lopez-Calcerrada S, Mlody B, Otto M, Wu H, Richter Y, Roth P, Anand R, Kulka LAM, Meierhofer D, Glazar P, Legnini I, Telugu NS, Hahn T, Neuendorf N, Miller D, Böddrich A, Polzin A, Mayatepek E, Diecke S, Olzscha H, Kirstein J, Ugalde C, Petrakis S, Cambridge S, Rajewsky N, Kühn R, Wanker EE, Priller J, Metzger J, Prigione A.

Abstract

Expansion of the glutamine tract (poly-Q) in the protein huntingtin (HTT) causes the neurodegenerative disorder Huntington's disease (HD). Emerging evidence suggests that mutant HTT (mHTT) disrupts brain development. To gain mechanistic insights into the neurodevelopmental impact of human mHTT, we engineered male induced pluripotent stem cells to introduce a biallelic or monoallelic mutant 70Q expansion or to remove the poly-Q tract of HTT. The introduction of a 70Q mutation caused aberrant development of cerebral organoids with loss of neural progenitor organization. The early neurodevelopmental signature of mHTT highlighted the dysregulation of the protein coiled-coil-helix-coiled-coil-helix domain containing 2 (CHCHD2), a transcription factor involved in mitochondrial integrated stress response. CHCHD2 repression was associated with abnormal mitochondrial morpho-dynamics that was reverted upon overexpression of CHCHD2. Removing the poly-Q tract from HTT normalized CHCHD2 levels and corrected key mitochondrial defects. Hence, mHTT-mediated disruption of human neurodevelopment is paralleled by aberrant neurometabolic programming mediated by dysregulation of CHCHD2, which could then serve as an early interventional target for HD.

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Contribution: establishment, generation, and preparation of engineered iPSC-derived midbrain organoids for immunohistochemistry and single-cell RNA sequencing. Preparation of material and methods and figure design for the manuscript.

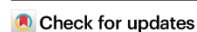


Mutant huntingtin impairs neurodevelopment in human brain organoids through CHCHD2-mediated neurometabolic failure

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Expansion of the glutamine tract (poly-Q) in the protein huntingtin (HTT) causes the neurodegenerative disorder Huntington's disease (HD). Emerging evidence suggests that mutant HTT (mHTT) disrupts brain development. To gain mechanistic insights into the neurodevelopmental impact of human mHTT, we engineered male induced pluripotent stem cells to introduce a biallelic or monoallelic mutant 70Q expansion or to remove the poly-Q tract of HTT. The introduction of a 70Q mutation caused aberrant development of cerebral organoids with loss of neural progenitor organization. The early neurodevelopmental signature of mHTT highlighted the dysregulation of the protein coiled-coil-helix-coiled-coil-helix domain containing 2 (CHCHD2), a transcription factor involved in mitochondrial integrated stress response. CHCHD2 repression was associated with abnormal mitochondrial morphodynamics that was reverted upon overexpression of CHCHD2. Removing the poly-Q tract from HTT normalized CHCHD2 levels and corrected key mitochondrial defects. Hence, mHTT-mediated disruption of human neurodevelopment is paralleled by aberrant neurometabolic programming mediated by dysregulation of CHCHD2, which could then serve as an early interventional target for HD.

Huntington's disease (HD) is a rare neurodegenerative disorder caused by inherited defects in the gene Huntingtin (*HTT*) encoding for the protein HTT. The mutant *HTT* (*mHTT*) gene exhibits an abnormal (>35) CAG expansion resulting in production of elongated repeats of glutamine (Q) in the poly-Q tract of the protein that could in turn become more prone to misfolding and intracellular aggregation^{1,2}. HD is currently incurable and the mechanisms underlying the neurodegenerative process are not fully understood.

An increasing amount of evidence points towards impaired brain development in HD. In fact, although wild-type (WT) HTT is present in several tissues, it is expressed at the highest level in the brain, even before the completion of neuronal maturation³. In mice, targeted HTT disruption is embryonic lethal^{4,5}, brain-specific HTT inactivation leads

to progressive neuronal defects⁶, and extensive HTT reduction fails to support normal brain development⁷. WT HTT may thus exert a physiological role in neurodevelopment, including axonal transport⁸, synapse development⁹, neural rosette formation¹⁰, neuronal migration¹¹, as well as regulation of neural progenitor cells (NPCs) and neurogenesis¹².

In accordance with a developmental component in HD, mHTT with a poly-Q tract longer than 60 causes severe juvenile forms of HD, which recapitulate features associated with neurodevelopmental disorders¹³. Even individuals with non-juvenile forms of HD can develop signs of impaired brain growth during childhood before the occurrence of clinical manifestation^{14–16}. In mice, mHTT disrupts the division of cortical progenitors¹⁷, and temporally-limited expression of

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Article

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mHTT in early life is sufficient to recapitulate the disease phenotypes¹⁸. Importantly, the analysis of human fetuses carrying mHTT demonstrated similar neurodevelopmental defects, with diminished numbers of proliferating NPCs and higher number of NPCs that prematurely enter neuronal lineage specification¹⁹.

An effective model system for dissecting the neurodevelopmental aspects of HD is represented by human induced pluripotent stem cells (iPSCs). The transcriptional signature of neurons differentiated from iPSC models of HD pointed toward a dysregulation of neurodevelopmental genes^{20–22}. Cerebral organoids carrying mHTT exhibited aberrant organization and specification of NPCs, with immature ventricular zones²³. The CAG repeat length could thus regulate the balance between NPC expansion and differentiation in cerebral organoids²⁴. Micro-patterned neuruloids consisting of NPCs, neural crest, sensory placode and epidermis showed disrupted self-organization in the presence of mHTT, indicating faulty neurodevelopment²⁵.

To gain mechanistic insights into how mHTT impacts human brain development, we generated brain organoids from engineered isogenic iPSC lines in which we introduced mHTT (70Q) in one or both *HTT* alleles, or we eliminated the poly-Q stretch completely from the *HTT* gene. We found that biallelic 70Q introduction disrupts the development of brain organoids (unguided cerebral organoids and region-specific cortical organoids and midbrain organoids) causing defective NPC organization despite seemingly unaffected neuronal presence. To identify mechanisms regulating early developmental defects caused by mHTT, we focused on shared signatures across undifferentiated iPSCs and neural committed cells. We identified the mitochondrial protein coiled-coil-helix-coiled-coil-helix domain containing 2 (CHCHD2) as a top dysregulated factor. In accordance to the known function of CHCHD2 in mitochondrial integrated stress response (mISR)²⁶, neural cells carrying mHTT developed a mISR signature with disruption of mitochondrial dynamics, cristae morphology, and bioenergetics, with specific complex IV alteration and increased energy expenditures at rest, a condition known as hypermetabolism. In-frame elimination of the poly-Q tract in *HTT* reverted key defects in CHCHD2 expression and mitochondrial morpho-dynamics. In HD patient-derived neuronal cultures, CHCHD2 belonged to a network of factors associated with axon guidance, Hippo signaling, and mISR, and its down-regulation impaired neurite outgrowth capacity. Additionally, AAV-mediated overexpression of CHCHD2 in mutant NPCs rescued the mitochondrial phenotype. Hence, mHTT is linked to mitochondrial dysfunctions in early neural cells through the dysregulation of CHCHD2, which could potentially contribute to altering the neurodevelopment process in humans.

Results

mHTT impairs early neural progenitor organization in human cerebral organoids

We engineered iPSCs with a healthy genomic background (WT/WT) using CRISPR/Cas9^{27,28}. The edited region within the *HTT* gene entailed both the CAG repeat stretch (poly-Q in the protein) and the CCG repeat stretch (poly-P in the protein) (Fig. 1a, Supplementary Fig. 1a). As template, we used an expanded tract composed of 70 CAG/CAA repeats mimicking mutations observed in patients. The resulting engineered iPSC lines were: i) WT/70Q carrying the expanded CAG tract only on one allele, ii) 70Q/70Q carrying the expanded CAG tract on both alleles, iii) 0Q/0Q with an in-frame deletion that eliminated the CAG and CCG stretches (Fig. 1a, b). We confirmed the successful editing by sequencing, PCR, and immunoblotting (Fig. 1c, Supplementary Fig. 1b, c, Supplementary Fig. 1e–h). The set of isogenic iPSC lines exhibited a normal karyotype (Supplementary Fig. 1i) and displayed no modification of top computationally predicted off-target sites (Supplementary Fig. 1d).

To assess the impact of mHTT on human brain development, we focused on the line 70Q/70Q, in which WT *HTT* was absent. In fact,

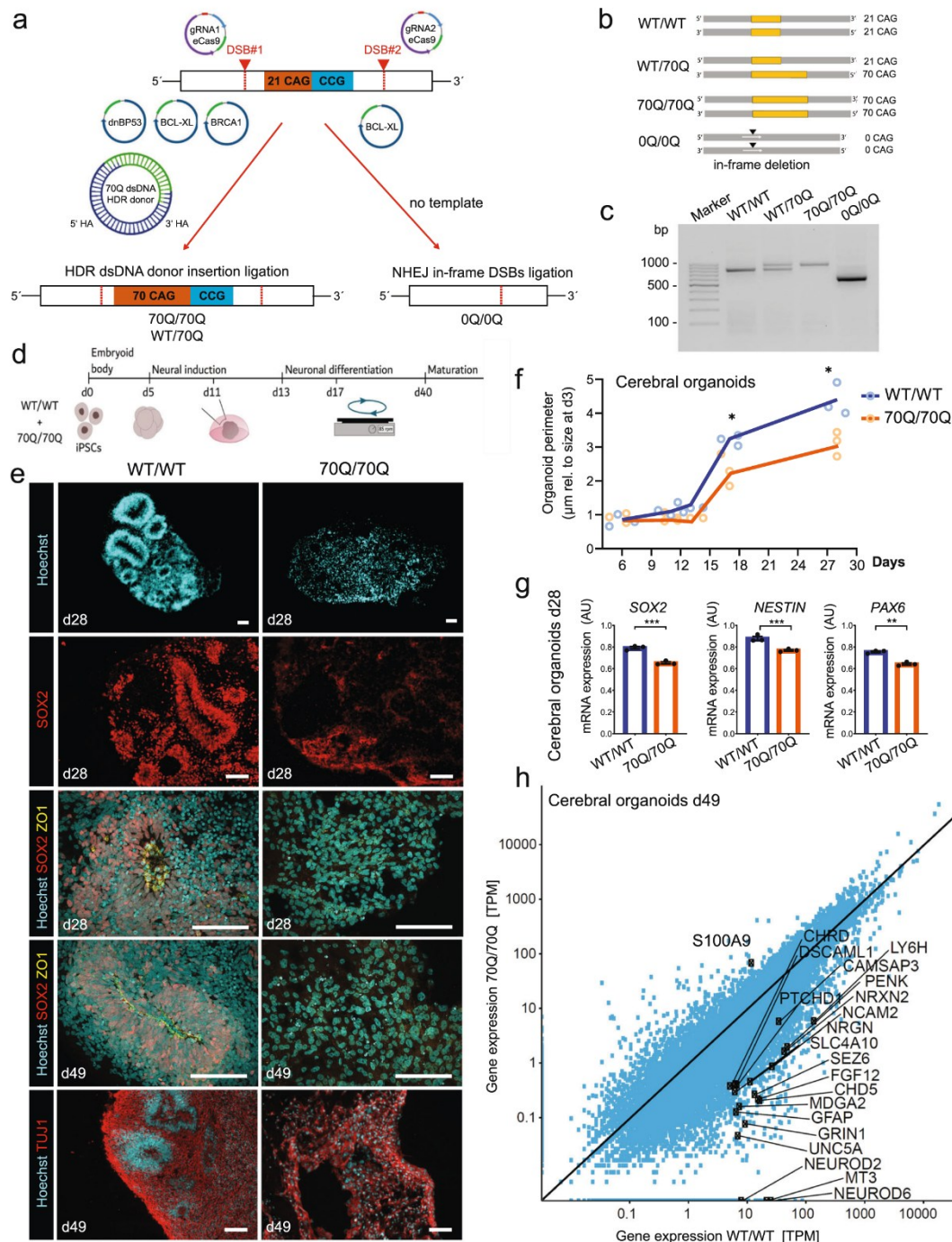
while heterozygous cells with expanded poly-Q tract have previously been used for the investigation of mHTT pathogenesis, to our knowledge a homozygous model of mHTT has not yet been studied using iPSCs. Hence, exploring the consequences of homozygous 70Q/70Q may allow us to shed light on the impact of mHTT on human neurodevelopment in the absence of potential compensatory effects exerted by WT *HTT*.

Using this homozygous mutant model (70Q/70Q) and its isogenic control (WT/WT), we applied an unguided cerebral organoid differentiation protocol (Fig. 1d). Cerebral organoids carrying mHTT (70Q/70Q) displayed significant disruption in cellular organization, including lack of ventricular zone-like neurogenic zones (Fig. 1e). NPCs appeared particularly impaired, as demonstrated by the reduced presence and spatial disorganization of cells positive for NPC markers SOX2 and FOXG1, and evident disruption of the tight junction marker ZO1 (Fig. 1e, Supplementary Fig. 2a). In accordance to features of neurodevelopmental impairment, cerebral organoids carrying mHTT displayed an overall reduced growth rate compared to WT organoids (Fig. 1f). Transcriptional analysis of cerebral organoids confirmed lower expression of progenitor markers (Fig. 1g). Additionally, the great majority of genes associated with the GO term “nervous system development” were downregulated in mutant organoids (Fig. 1h). Despite these alterations in the progenitor population, we could still detect neuronal cells in 70Q/70Q cerebral organoids, as indicated by the presence of markers TUJ1, MAP2, and SMI32 (Fig. 1e, Supplementary Fig. 2a, b). These findings are in line with previous data of mHTT-carrying human fetuses reporting the loss of proliferating NPCs, specific defects in ZO1 expression, and premature neuronal differentiation¹⁹.

We next aimed to dissect the impact of mHTT on development of different brain regions and generated guided region-specific brain organoids (Fig. 2a). Neuroanatomical studies suggest that neurodegeneration in HD individuals is not limited to striatum but also affect brainstem and neocortex regions²⁹. Therefore, we focused on cortical organoids and midbrain organoids. The presence of mHTT diminished the size development of both cortical and midbrain organoids (Fig. 2b, c). In both region-specific brain organoids, the overall size appeared altered already at relatively early stages, even before 10 days of differentiation (Fig. 2b, c, Supplementary Fig. 3a–d). We next checked for presence of toxic HTT aggregates during brain organoid development. We could not find significant presence of large-size HTT protein conformations after 70 days of in vitro culture of cortical organoids or midbrain organoids (Supplementary Fig. 2c, d).

To delve deeper into the impact of mHTT on neural progenitors and maturing neurons within developing brain organoids, we focused on midbrain organoids. In the protocol we used³⁰, midbrain organoids were smaller than cortical organoids and exhibited a simpler organization, with concomitant presence of neuronal and progenitor markers (Fig. 2d). We performed single-cell RNA-sequencing of 70Q/70Q and WT/WT midbrain organoids at 35 days. We identified four main cell populations: i) proliferative progenitors, ii) progenitors, iii) maturing neurons, iv) mature neurons (Fig. 2e, Supplementary Data 1). These four populations expressed specific gene markers, such as TOP2A for proliferative progenitors, PTPRZ1 for progenitors, ROBO3 for maturing neurons, and MAPT for mature neurons (Fig. 2g, Supplementary Data 1). In agreement with the findings seen in unguided cerebral organoids (Fig. 1e), 70Q/70Q midbrain organoids exhibited a marked reduction of “progenitors” and “proliferative progenitors” populations, despite the continued presence of mature neurons (Fig. 2e, f, Supplementary Fig. 3e, f).

Collectively, these data suggest that mHTT causes neurodevelopmental defects that occur early on during development and may specifically impact neural progenitors.



CHCHD2 is a top dysregulated gene within the early neurodevelopmental signature of mHTT

We next aimed to transcriptionally dissect the mechanisms underlying the early neurodevelopment defects caused by mHTT. PolyQ tract motifs found in transcription factors can act as transcriptional regulating domains by facilitating the binding between transcription factors and transcriptional regulators³¹. To thoroughly analyze mHTT's

transcriptional impact we conducted total RNA sequencing of 70Q/70Q and WT/WT at different time points of neurodevelopment: i) iPSCs, ii) NPCs, iii) cerebral organoids at day 28 (for early neurogenesis), iv) cerebral organoids at day 49 (for late neurogenesis and astrogenesis) (Fig. 3a, Supplementary Data 2).

We focused on genes that were dysregulated early and persistently throughout the differentiation trajectory. We identified a

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Fig. 1 | Engineered iPSCs carrying mHTT give rise to neurodevelopmentally impaired cerebral organoids. **a** Genome editing approach in control iPSCs (WT/WT) using two double-strand breaks (DSB) sites to modify the HTT genomic region encompassing the CAG/CAA and CCG repeats stretches. To generate iPSCs carrying elongated CAG in one allele (70Q/WT) or in both alleles (70Q/70Q), we promoted homology direct repair (HDR) (with plasmids BCL-XL and BRCA1), inhibited non-homologous end joining (NHEJ) (with plasmid dnBP53), and provided a HDR donor dsDNA plasmid carrying 70Q repeats and homology arms. We harnessed NHEJ to obtain iPSCs with in-frame deletion of the CAG/CCG region (0Q/0Q). **b** Overview of the engineered isogenic iPSC lines. **c** PCR analysis of HTT in the isogenic iPSC lines. Data were repeated in three independent experiments. **d** Schematics of the protocol to generate unguided cerebral organoids from isogenic iPSC lines WT/WT and 70Q/70Q. **e** Immunostaining in cerebral organoids at day 28 and 49 showing

defective cytoarchitecture and neural progenitor cell (NPC) organization in 70Q/70Q. Data were repeated in three independent experiments. Scale bar: 100 μ m. **f** Growth rate of cerebral organoids with respect to the initial perimeter size measured at day 3. $n = 3$ independent organoid differentiations (dots) per line. Each dot represents the average size of all cerebral organoids measured in one biological replicate. $^*p < 0.05$ WT/WT vs. 70Q/70Q; unpaired two-tailed Welch t test. **g** qPCR analysis of NPC markers in cerebral organoids at day 28. Mean $\pm$ s.e.m.; $n = 3$ independent biological replicates (dots) per line; $^{***}p < 0.001$ WT/WT vs. 70Q/70Q; unpaired two-tailed t test (AU=arbitrary units). Four organoids were pooled for each individual RNA isolation. **h** Gene expression analysis of cerebral organoids at day 49 highlighting the genes belonging to the GO term “nervous system development” (GO:0007399).

consistent expression signature comprising 47 genes that were either down- or up-regulated in 70Q/70Q compared to WT/WT across all developmental stages (Fig. 3a, Supplementary Fig. 4a, b, Supplementary Data 3). Within this signature, the most downregulated gene in mHTT-expressing cells was coiled-coil-helix-coiled-coil-helix domain containing 2 (CHCHD2) (Fig. 3b). Other downregulated factors included genes involved in development (GBX2, PAX7, PAX8, LHX1, LMX1B, BARX1), neuronal development (POUF33, POUF3F2, TAF9B, EN2), and glutamate metabolism (SLC1A2, GAD1, GAD2) (Fig. 3b, Supplementary Fig. 4b). Upregulated factors in mHTT condition were genes associated with detoxification (GSTM1, IAH1), protein translation (EIFIAY, KDM5D), and protein degradation (USP9Y). In addition to this common signature, genes involved in nervous system development were downregulated in NPCs and cerebral organoids carrying mHTT (Supplementary Fig. 4d).

We performed untargeted proteomics of iPSCs and NPCs to determine whether any of the observed transcriptional changes induced by mHTT were recapitulated at the protein level. We identified metabolism-related proteins dysregulated in both iPSCs (46 metabolic proteins out 321) and NPCs (13 out of 484) (Fig. 3c, d, Supplementary Data 4, 5). Among these proteins, CHCHD2 was significantly downregulated in 70Q/70Q NPCs compared to WT/WT NPCs (Fig. 3d). Given that transcriptomics and proteomics identified CHCHD2 as dysregulated upon 70Q introduction, we inspected the transcription level of the whole family of coiled-coil-helix-coiled-coil-helix (CHCH) domain-containing proteins. These are proteins imported into the mitochondrion that have been suggested to play a role in neurodegenerative diseases^{32,33}. Although other CHCH family members were dysregulated by mHTT, CHCHD2 appeared most significantly affected in both iPSCs and NPCs carrying the elongated CAG repeat in both alleles (70Q/70Q) or in only one allele (WT/70Q) (Fig. 3e). We also found a similar dysregulation of CHCHD2 in previously published datasets of neuruloids carrying mHTT²⁵ (Fig. 3e).

To validate the decreased expression of CHCHD2 in cells carrying mHTT, we performed immunostaining for CHCHD2 in cerebral organoids and NPCs and applied a semi-automated pipeline to quantify the expression of CHCHD2. Using this approach, we confirmed a decreased level of CHCHD2 protein in cerebral organoids and NPCs carrying mHTT (70Q/70Q) compared to WT/WT (Fig. 3f, g). NPCs carrying mHTT in only one allele (WT/70Q) also showed an overall reduced abundance of CHCHD2, although this difference was not statistically significant (Fig. 3g). This may be attributed to the insignificant reduction of HTT amount that we observed in the WT/70Q line (Supplementary Fig. 1c). The elimination of both poly-Q and poly-P tracts (0Q/0Q) significantly increased CHCHD2 protein expression in NPCs (Fig. 3g). We further confirmed these results using immunoblot analysis of CHCHD2, which showed increased expression in 0Q/0Q NPCs and reduced expression in 70Q/70Q NPCs when compared to WT/WT NPCs (Supplementary Fig. 4e, f).

In summary, mHTT leads to downregulation of CHCHD2 during early neurodevelopment and the presence of CHCHD2 can be restored

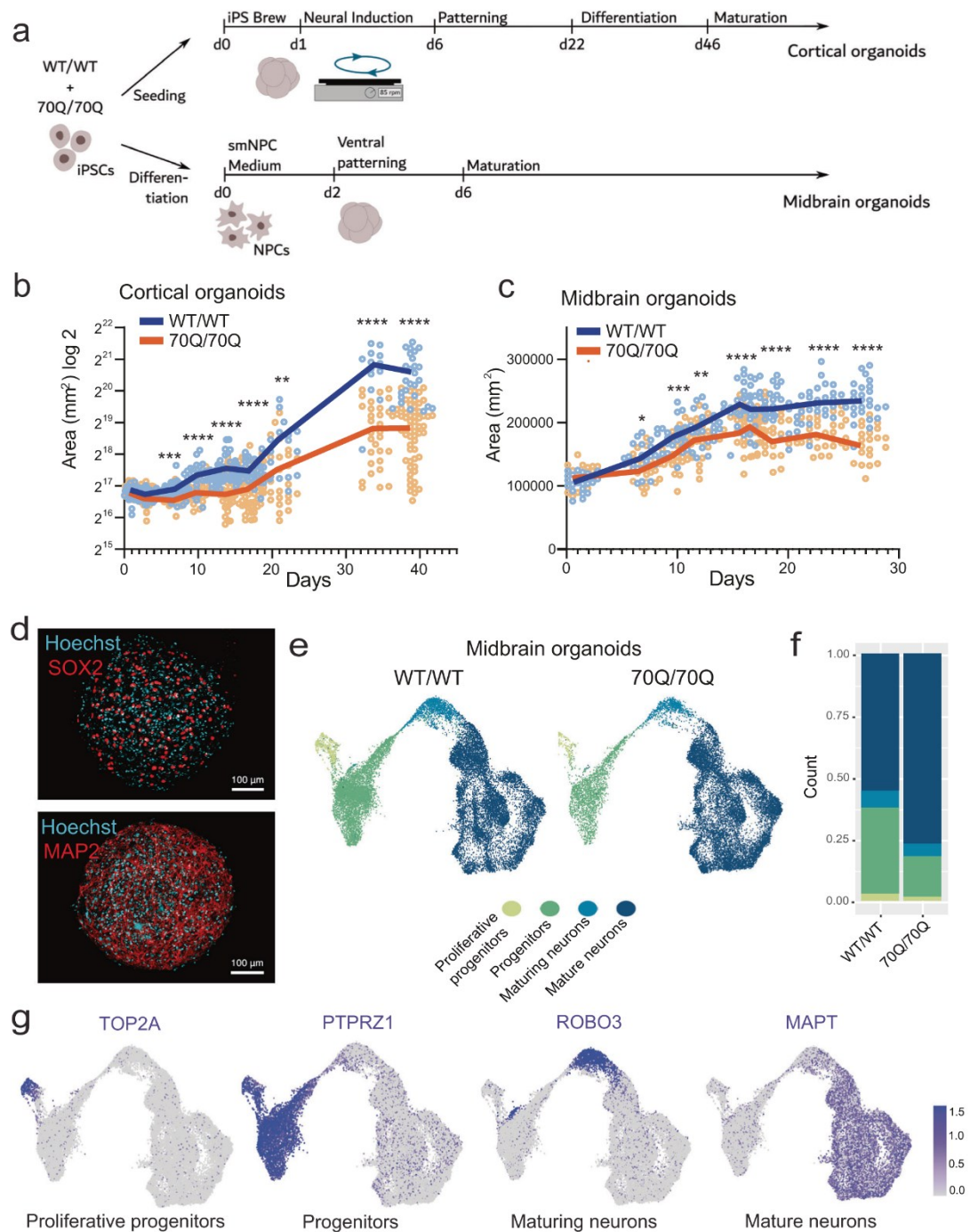
by eliminating the CAG/CCG region, suggesting a potential role for CHCHD2 in HD neuropathology.

CHCHD2 dysregulation by mHTT is associated with a mISR signature and defective mitochondrial morpho-dynamics

We next investigated the downstream consequences associated with the dysregulation of CHCHD2. CHCHD2 is known to be imported into the mitochondria and may also act as a transcription factor by translocating to the nucleus³⁴. It was indeed listed among nucleic acid binding genes in our transcriptomics dataset (Supplementary Fig. 4c). Using semi-automated quantification of immunostained images, we confirmed that both expression and nuclear localization of CHCHD2, as indicated by co-localization with the DNA stain Hoechst, were diminished in NPCs and cerebral organoids (COs) carrying mHTT 70Q/70Q (Fig. 4a, b). Midbrain organoids carrying 70Q/70Q also displayed lower CHCHD2 presence (Supplementary Fig. 5a).

CHCHD2 plays a role in mitochondrial integrated stress response (mISR) and mitochondrial dynamics²⁶. Accordingly, genes associated with mISR were dysregulated upon 70Q introduction (Fig. 4c). This was particularly evident for NPCs (70Q/70Q and WT/70Q), where mISR-related genes were significantly upregulated compared to WT/WT (e.g., ATF3, ATF4, DDIT3, CHAC1, PCK2). mISR dysregulation was present also in cerebral organoids at day 49 and HD neuruloids, although to a lower extent. In addition, mHTT altered the expression of genes regulating mitochondrial dynamics in mutant NPCs and cerebral organoids, with upregulation of genes related to mitochondrial fusion (e.g., FIS1, MNF1, MNF2) and changes in the expression of genes associated with mitochondrial fission (OMAL, MFF, DNML1) (Fig. 4c). In conditions with impaired mitochondrial oxidative phosphorylation (OXPHOS), mISR is associated with dysregulation of mitochondrial quality control and mitochondrial dynamics, and cellular senescence³⁵. Indeed, genes related to mitochondrial quality control, mitochondrial biogenesis, and senescence were affected by mHTT, with upregulation of quality control genes in NPCs (70Q/70Q and WT/70Q) and cerebral organoids (70Q/70Q) (e.g., YMEIL1, LONP1, LONP2, HSPA1) (Supplementary Fig. 5e).

To gain additional insights into the impact of mHTT in neural progenitors and the potential relationship with CHCHD2 dysregulation, we carried out long-read transcriptomics in 70Q/70Q NPCs and WT/WT NPCs. Since alternative splicing plays a central role during neurogenesis³⁶ and is particularly disrupted in HD³⁷, we aimed to discover potential differences in NPC isoform usage caused by mHTT (Supplementary Fig. 5b). We identified unique isoforms (128712 in WT/WT and 152214 in 70Q/70Q) but observed no differences in the overall number of isoforms per gene in mutant NPCs compared to WT (Supplementary Fig. 5c, d). Nonetheless, we observed specific different isoform patterns for genes involved in mitochondrial dynamics, including NRF1, YMEIL1, DNML1, and MFN2 (Fig. 4d). Changes in DNML1, encoding for a protein also known as dynamin-related protein 1 (DRP1) are of particular interest, since this protein has been found to



interact with HTT^{38,39}, and its transcript variants can influence the state of mitochondrial dynamics⁴⁰.

Altered expression and variant patterns of genes involved in mitochondrial dynamics suggested possible alterations in mitochondrial morpho-dynamics. We thus evaluated mitochondrial dynamics and mitochondrial network morphology in cerebral organoids based on the expression pattern of the mitochondrial outer membrane

marker TOM20 (Fig. 4e)⁴¹. TOM20 is a protein located in outer mitochondrial protein that is typically employed for visualizing mitochondrial structures. We found that mutant cerebral organoids exhibited elevated mitochondrial footprint, which we defined as the concomitant existence of fragmented mitochondria and abnormally large mitochondrial network structures (Fig. 4e, white arrows), leading to an overall increase in the intensity of TOM20-positive signals

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Fig. 2 | mHTT compromises development and neural progenitor population in region-specific brain organoids. **a** Schematics of the protocol to generate guided region-specific brain organoids from iPSC lines WT/WT and 70Q/70Q. For cortical organoids, we started the differentiation from iPSCs, for midbrain organoids we started from NPCs. **b** Growth curve of cortical organoids. Dots represent individual organoids over at least two independent experiments. We compared organoid size at defined time points (day 1, 2, 7, 10, 14, 17, 21, 34, 39): *** $p < 0.001$, **** $p < 0.0001$ WT/WT vs. 70Q/70Q; two-tailed Mann-Whitney U test. **c** Growth curve of midbrain organoids. Dots represent individual organoids over at least two independent experiments. We compared organoid size at defined time points (day 1, 7, 10, 12, 16, 17, 19, 23, 27): *** $p < 0.001$, **** $p < 0.0001$ WT/WT vs. 70Q/70Q; two-tailed Mann-Whitney U test. **d** Midbrain organoids stained for neural progenitor marker SOX2

and neuronal marker MAP2 to show their uniform distribution within individual organoids. Data were repeated in two independent experiments. Scale bar: 100 μ m. **e** Single-cell analysis of midbrain organoids. Uniform manifold approximation and projection (UMAP) showing the overall cell composition of WT/WT and 70Q/70Q midbrain organoids. Each sample was sequenced in three biological replicates, with each replicate containing around 48 individual midbrain organoids. Shown here are merged UMAP images for all three replicates for WT/WT and three replicates for 70Q/70Q. **f** Quantification of the four annotated cell populations in WT/WT and 70Q/70Q midbrain organoids. **g** Distribution of exemplary markers for each of the four cell populations composing the midbrain organoids (see Supplementary Data 1).

(Fig. 4f). Both small mitochondrial structures and large mitochondrial structures appeared increased in mutant cerebral organoids (Supplementary Fig. 5f). The overall increase in TOM20 levels was more evident in mutant cerebral organoids at day 49 compared to day 28, suggesting that defects in mitochondrial dynamics might become more severe over time (Fig. 4f). Using a semi-automated quantification of immunostaining images, we confirmed higher relative levels of TOM20 protein cerebral organoids carrying 70Q/70Q compared to WT/WT and in 70Q/70Q NPCs compared to WT/WT NPCs. The TOM20 increase did not reach significance in NPCs with WT/70Q (Fig. 4g). These latter findings are consistent with the observation that WT/70Q did not show downregulation of CHCHD2 (Fig. 3g) or reduction of HTT levels (Supplementary Fig. 1c). At the same time, elimination of the poly-Q/poly-P region was sufficient to repress the abnormal TOM20 increase in NPCs (Fig. 4g).

Taken together, neural cells carrying mHTT exhibited CHCHD2 dysregulation and developed a mISR signature and aberrant mitochondrial morpho-dynamics, and these features could be reverted upon elimination of the CAG/CCG repeat region.

mHTT in neural progenitors causes aberrant metabolic programming and mitochondrial complex IV defects

Following the identification of defects in mitochondrial morpho-dynamics caused by mHTT, we sought to investigate mitochondrial morphology in NPCs by electron microscopy (Fig. 5a). We did not observe significant changes in the size of mitochondria in 70Q/70Q NPCs compared to WT/WT NPCs (Supplementary Fig. 5g). However, elimination of the CAG/CCG region led to an increase in mitochondrial roundness and mitochondrial area in 0Q/0Q NPCs compared to WT/WT NPCs (Supplementary Fig. 5g). Whether these changes could have detrimental consequences remains to be determined. A specific defect that we observed in 70Q/70Q NPCs was the presence of mitochondrial cristae oriented longitudinally with respect to the outer membrane (Fig. 5a, black arrowhead; Fig. 5b) instead of transversally (Fig. 5a, black arrows; Fig. 5b). Such mitochondrial cristae alteration has been suggested to develop as a consequence of mHTT^{42,43}. The mitochondrial cristae defect was reverted in 0Q/0Q NPCs, in which cristae direction appeared to be transverse as in the case of WT (Fig. 5a, b).

To elucidate the functional metabolic consequences associated with the observed changes in mitochondrial morpho-dynamics caused by mHTT, we carried out functional metabolic pathway analyses based on our proteomics dataset. Such an approach was previously carried out to pinpoint a disruption in mitochondrial metabolism in cerebral organoids from autism spectrum disorders⁴⁴. The kinetic model used comprises the major cellular metabolic pathways of energy metabolism in neural cells, including ion membrane transport and mitochondrial membrane potential (Supplementary Data 7). Introduction of 70Q mutation in NPCs led to increased lactate production and diminished mitochondrial oxidation (Fig. 5c). Accordingly, gene set enrichment analysis of proteomics indicated that 70Q/70Q NPCs exhibited lower OXPHOS and higher glycolysis/gluconeogenesis compared to WT/WT NPCs (Fig. 5d). NPCs carrying mHTT also showed

higher glucose utilization at rest (Fig. 5e). This feature of elevated resting energy expenditure is also known as hypermetabolism, a condition developing upon impaired OXPHOS function in primary mitochondrial diseases that is associated with mISR activation³⁵. We next investigated the ratio of NAD/NADH and mitochondrial NAD/NADH, crucial indicators of metabolic and redox homeostasis⁴⁵. Both ratios appeared reduced in mutant NPCs compared to wild-type, indicating the presence of mitochondrial impairment and reductive stress (Fig. 5f).

Some of the metabolic changes detected in mutant NPCs were already present in mutant iPSCs, including increased lactate production coupled with reduced mitochondrial oxidation, and increased substrate utilization at rest (Supplementary Fig. 6a–c). Mutant iPSCs also exhibited a reduction in ATP/ADP ratio and maximal ATP production (Supplementary Fig. 6f, g). In contrast to NPCs however, the ratios of NAD/NADH and mitochondrial NAD/NADH were less significantly altered in mutant iPSCs compared to WT/WT iPSCs (Supplementary Fig. 6d, e). We speculate that this could be due to the fact that iPSCs predominantly rely on glycolytic metabolism, while NPCs already start to depend on OXPHOS as a consequence of initiation of neural commitment^{46,47}. Hence, the functional consequences of defective metabolic programming may become more evident when cells acquire higher mitochondrial capacity during neurogenesis.

We next assessed whether these metabolic defects induced by mHTT could impact the protein organization of the mitochondrial respiratory chain proteins. With SDS-PAGE, we identified a specific decrease in the levels of complex IV proteins in 70Q/70Q NPCs compared to WT/WT NPCs that was restored in 0Q/0Q NPCs (Fig. 5g, h). This was in agreement with the proteomics findings in NPCs, where the expression of the complex IV subunit COX7A2 was among the most downregulated proteins in mutant NPCs compared to wild-type NPCs (Fig. 3d). Of note, COX7A2 is a key determinant for the structural and functional organization of the mitochondrial respiratory chain⁴⁸. Blue native PAGE analysis further highlighted a reduction of complex IV assembly in 70Q/70Q NPCs compared to WT/WT NPCs that was partially reverted in 0Q/0Q NPCs (Fig. 5i, j, Supplementary Fig. 6h). Accordingly, transcriptional analysis confirmed the dysregulation of mitochondrial complex IV genes (e.g., COX7A2) and mitochondrial complex IV assembly factors (e.g., SCO2) in NPCs, cerebral organoids and neurospheres carrying mHTT (Supplementary Fig. 6i, j).

Collectively, these findings suggest that mHTT caused hypermetabolism and defects in mitochondrial cristae and complex IV assembly that could be reversed by eliminating the CAG/CCG region. This bioenergetic impairment may start early during development upon initiation of neural commitment.

Identification of a CHCHD2-linked gene/protein/metabolite network in HD patient-derived neurons

We next sought to determine whether functional mitochondrial defects caused by mHTT could occur in human neurons in a cell-autonomous manner, independently of potential disruption of other cell types presented within cerebral organoids. Several publications

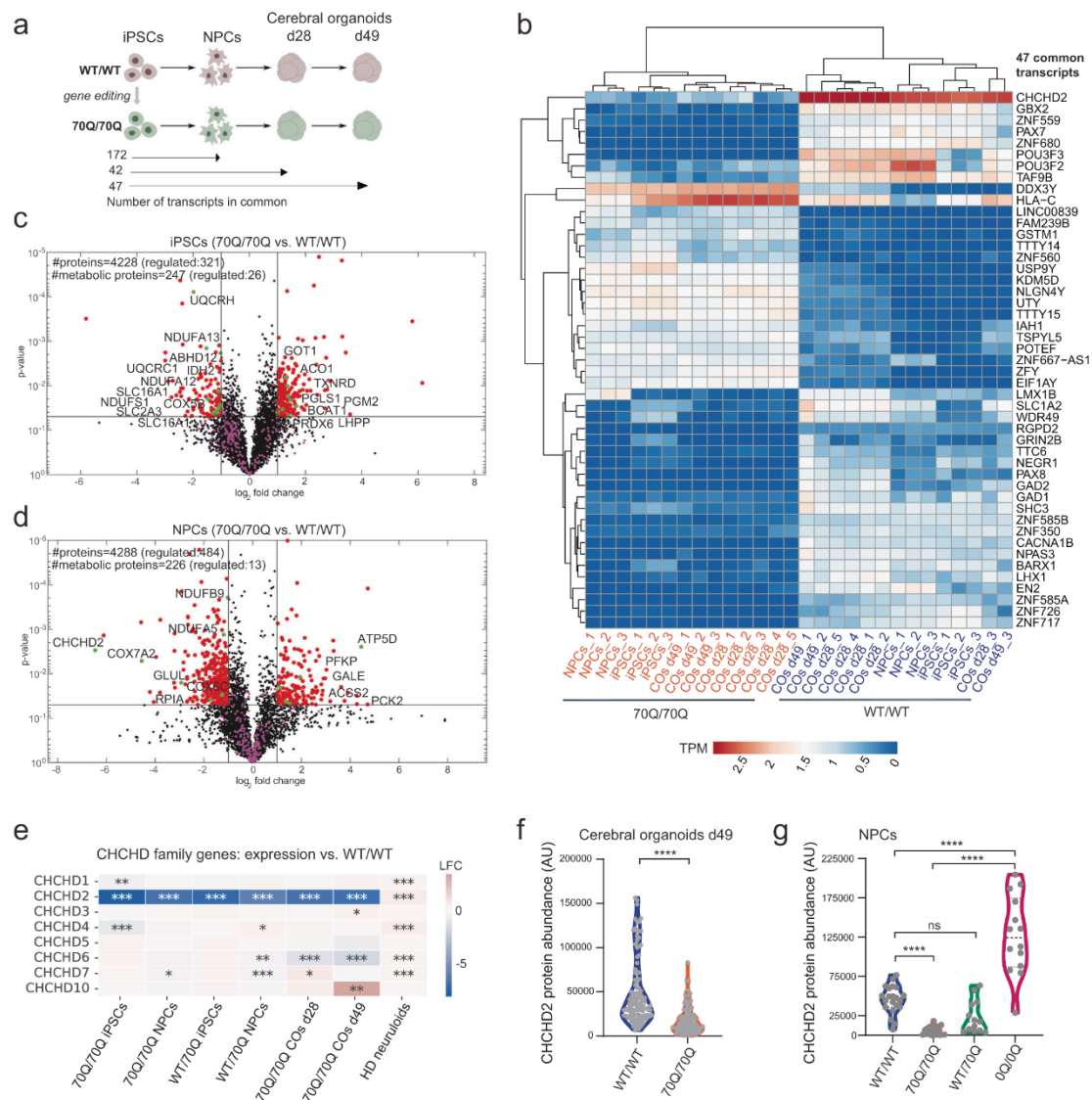
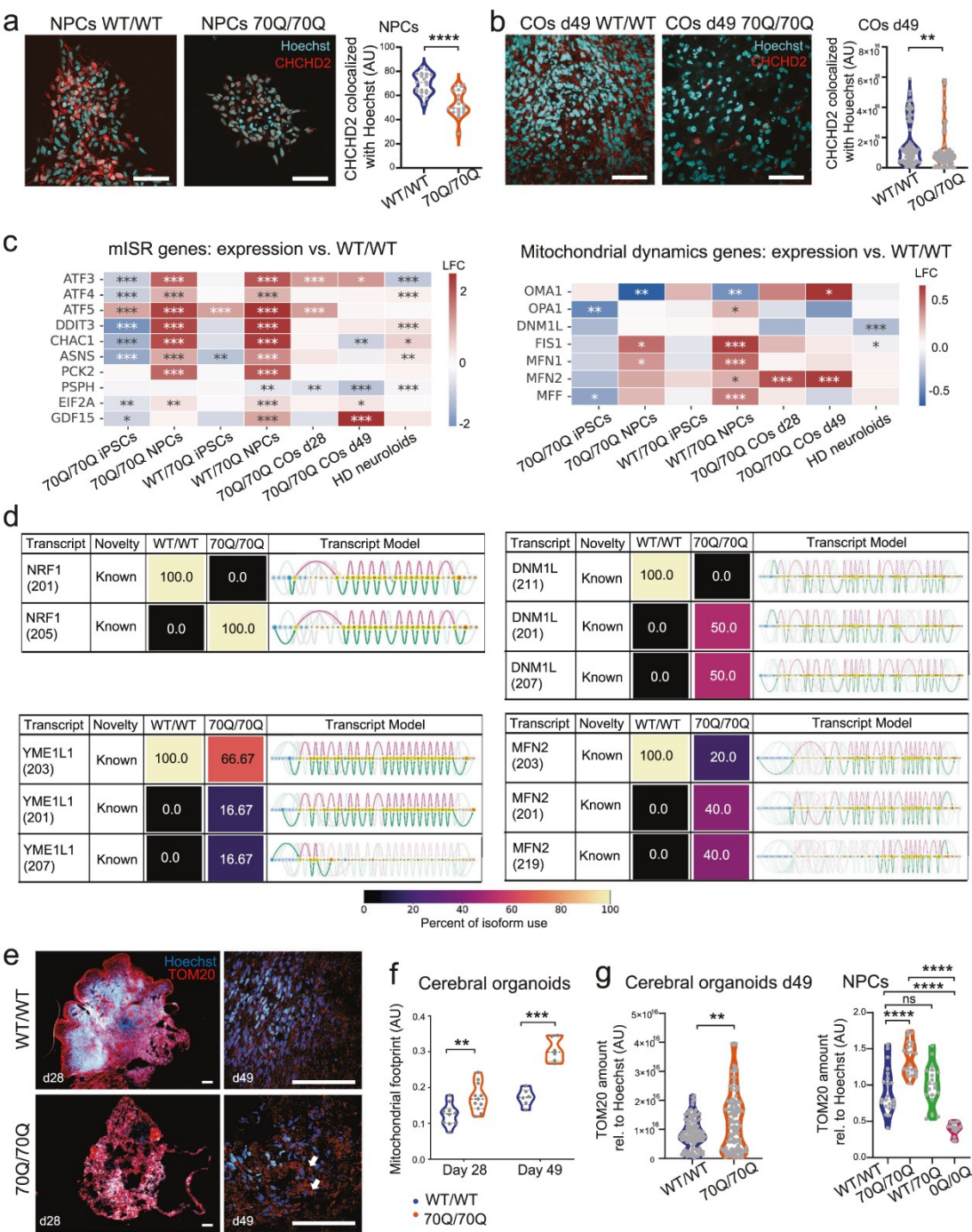


Fig. 3 | Omics analyses of mHTT-expressing cells across neurodevelopmental stages highlight the dysregulation of CHCHD2. **a** Schematic of RNA sequencing experimental set up for isogenic lines WT/WT and 70Q/70Q. Below are reported the number of transcripts uniquely in common across the neurodevelopmental stages (iPSCs, NPCs, cerebral organoids (COs) at day 23 and COs at day 49) (see Supplementary Data 2, 3). **b** Heatmap showing the 47 transcripts uniquely in common across the neurodevelopmental stages. TPM: transcripts per million. **c, d** Volcano plot of proteomic datasets in iPSCs and NPCs showing statistical significance (p value) versus magnitude of change ($\log_2$ fold change) based on two-sample two-tailed *t*-test with Benjamini-Hochberg (BH, FDR of 0.05) correction for multiple testing (see Supplementary Data 4–6). Red dots indicate the significantly differently regulated proteins (right quadrant: upregulated in 70Q/70Q; left

quadrant: downregulated in 70Q/70Q). Metabolic proteins were later used for the proteomic-driven functional metabolic analysis (see Supplementary Data 6). Green dots highlight regulated proteins that are explicitly named. **e** Heatmap of log-fold change (LFC) comparisons of differential gene expression of genes belonging to the CHCHD family. Data obtained from RNA sequencing of iPSCs, NPCs, and COs; **p* < 0.05, ***p* < 0.01, ****p* < 0.001; 70Q/70Q and WT/70Q vs. WT/WT, HD neuruloids vs. WT neuruloids²⁵; two-sided likelihood ratio test, without multiple comparison adjustments. **f, g** Quantifications of protein abundance of CHCHD2 based on immunostaining performed in cerebral organoids and NPCs. The amount of positive CHCHD2 signal per image was normalized to the Hoechst signal. 4*****p* < 0.0001, ns: not significant; two-tailed Mann-Whitney *U* test.

investigated iPSC-derived neurons carrying mHTT, but the various differentiation protocols employed typically resulted into a heterogeneous mixture of neuronal and glial population^{21,41}. Moreover, we wanted to make sure that our findings based on engineered cells reflected phenotypes actually occurring in patient-derived cells.

To address these aspects, we used three iPSC lines that we recently derived from individuals affected by HD: HD1 with WT/180Q⁴⁹ and HD2 and HD3 with WT/58Q and WT/44Q, respectively⁵⁰ (Fig. 6a). We compared the three HD iPSC lines to three iPSC lines derived from healthy control individuals (C1, C2, C3). To obtain a homogenous



population of neurons, we overexpressed the transcription factor Neurogenin 2 (NGN2)⁵¹. The conventional approach for NGN2 neuron derivation is based on direct reprogramming of iPSCs into neurons⁵¹. However, this approach is rather artificial, as it bypasses the NPC stage, and therefore the neuronal generation is not affected by processes influencing NPC physiology. Given that NPCs carrying mHTT exhibited functional defects, we opted for an approach that was instead more

physiological: we first generated NPCs and then applied NGN2 overexpression on the obtained NPCs (Fig. 6b). In this way, we reasoned that we may be able to assess the ability of cells carrying mHTT to generate neurons even in the presence of potential defects at the NPC stage. The constructs we used contained a GFP reporter and allowed derivation of homogeneous neuronal cultures expressing neuronal and synaptic markers (Supplementary Fig. 7a). Using this approach, we

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Fig. 4 | mISR and altered mitochondrial morpho-dynamics are associated with CHCHD2 dysregulation in mHTT-carrying neural cells. a, b Representative immunostaining and related quantifications of CHCHD2 protein colocalization with nuclear staining Hoechst in NPCs and in cerebral organoids (COs) at day 49. Dots represent individual images collected from at least two biological replicates for NPCs ($n =$ at least 35 individual images per sample) and COs ($n =$ at least 77 individual images per sample). $^{**}p < 0.01$, $^{****}p < 0.0001$; two-tailed Mann-Whitney U test. Scale bar: 100 μm . **c** Heatmap of log-fold change (LFC) comparisons of genes involved in mitochondrial integrated stress response (mISR) and mitochondrial dynamics. Data obtained from RNA sequencing of iPSCs, NPCs, and COs; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; 70Q/70Q and WT/70Q vs. WT/WT, HD neuruloids vs. WT neuruloids²⁵; two-sided likelihood ratio test, without multiple comparison adjustments. **d** Isoform usage in NPCs determined with long-read transcriptomics.

Percentage of use in WT/WT NPCs and 70Q/70Q NPCs and related transcript model are shown. **e, f** Representative immunostaining and related quantification of mitochondrial footprint (comprising both small and large mitochondrial structures) based on TOM20 signal in cerebral organoids from 70Q/70Q and WT/WT. Dots represent individual images collected over three independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$, unpaired two-tailed t test (AU = arbitrary units). Scale bar: 100 μm . **g** Quantifications of protein abundance of TOM20 based on immunostaining performed in cerebral organoids and NPCs. The amounts of positive TOM20 signal per image was normalized to Hoechst signal. Dots represent individual images collected from at least two biological replicates for NPCs ($n =$ at least 35 individual images per sample) and COs ($n =$ at least 77 individual images per sample) (AU = arbitrary units). $^{**}p < 0.01$, $^{****}p < 0.0001$, ns: not significant; two-tailed Mann-Whitney U test.

found that all patient-derived NPCs generated NGN2 neurons without visible differences in terms of efficiency and neuronal appearance compared to NGN2 neurons derived from control NPCs (Supplementary Fig. 7b, c).

We applied a multi-omics approach to NGN2 neurons derived from the three HD individuals and the three controls (Fig. 6b). Principal component analyses of transcriptomics, proteomics, and metabolomics showed distinctions between HD neurons and control neurons, although the distinction was less clear in the case of metabolomics due to higher variability in the control samples (Supplementary Fig. 7d, Supplementary Data 7–10). Nonetheless, it appeared that all HD neurons clustered closely in all omics irrespective of different polyQ lengths. These results suggest that the presence of mHTT alters the global expression pattern of human neurons in a cell-autonomous manner and regardless of the number of CAG repeats.

Upregulated genes in HD neurons were related to pathways involved in synaptic signaling and axon guidance, and to diseases associated with neurodevelopmental defects (Fig. 6c). Similarly, upregulated proteins in HD neurons were related to pathways and diseases involved in axon guidance, autophagy, mTOR, and neurodegeneration, including movement diseases and HD (Fig. 6d). DNML1 also appeared among these dysregulated proteins (Fig. 6d). Conversely, downregulated genes/proteins in HD neurons were associated with carbon metabolism, Hippo signaling, and muscular disorders (Supplementary Fig. 7e–h, Supplementary Data 7–10). Axon guidance appeared also among the downregulated pathways, suggesting that this process was particularly affected by mHTT (Supplementary Fig. 7e). Differentially altered metabolites in HD neurons grouped based on structural similarities included metabolites related to NAD and mitochondrial Acetyl-CoA and to lactate and lipid metabolism (Fig. 6e, Supplementary Fig. 7i, j, Supplementary Data 7–10). To validate the metabolomics data, we assessed the bioenergetic profiles of NGN2 neurons. HD neurons exhibited defective mitochondrial function, with diminished ATP production and maximal respiration (Fig. 6f, Supplementary Fig. 7k). At the same time, the glycolytic capacity of HD neurons was also compromised, as seen by significantly reduced basal glycolysis and lactate production (Fig. 6f, g). Altogether, the presence of mHTT in pure human neurons caused on one hand an overall defect in cellular bioenergetics, and on the other hand it led to aberrant expression of genes and proteins involved in neurodevelopment, axon guidance, and synaptic activity.

To gain additional insights into these changes and their correlation, we integrated the various omics datasets. First, we looked at protein co-expression networks. The biggest cluster of co-expressed proteins ($n = 272$) contained three members of the CHCH domain-containing proteins (CHCHD1, CHCHD2, and CHCHD5) (Supplementary Fig. 8a). Enrichment analysis for upstream transcription factors of these 272 co-expressed proteins highlighted components related to neurodevelopment and Hippo signaling, including SIN3A, and to human diseases such as developmental delay and mental retardation

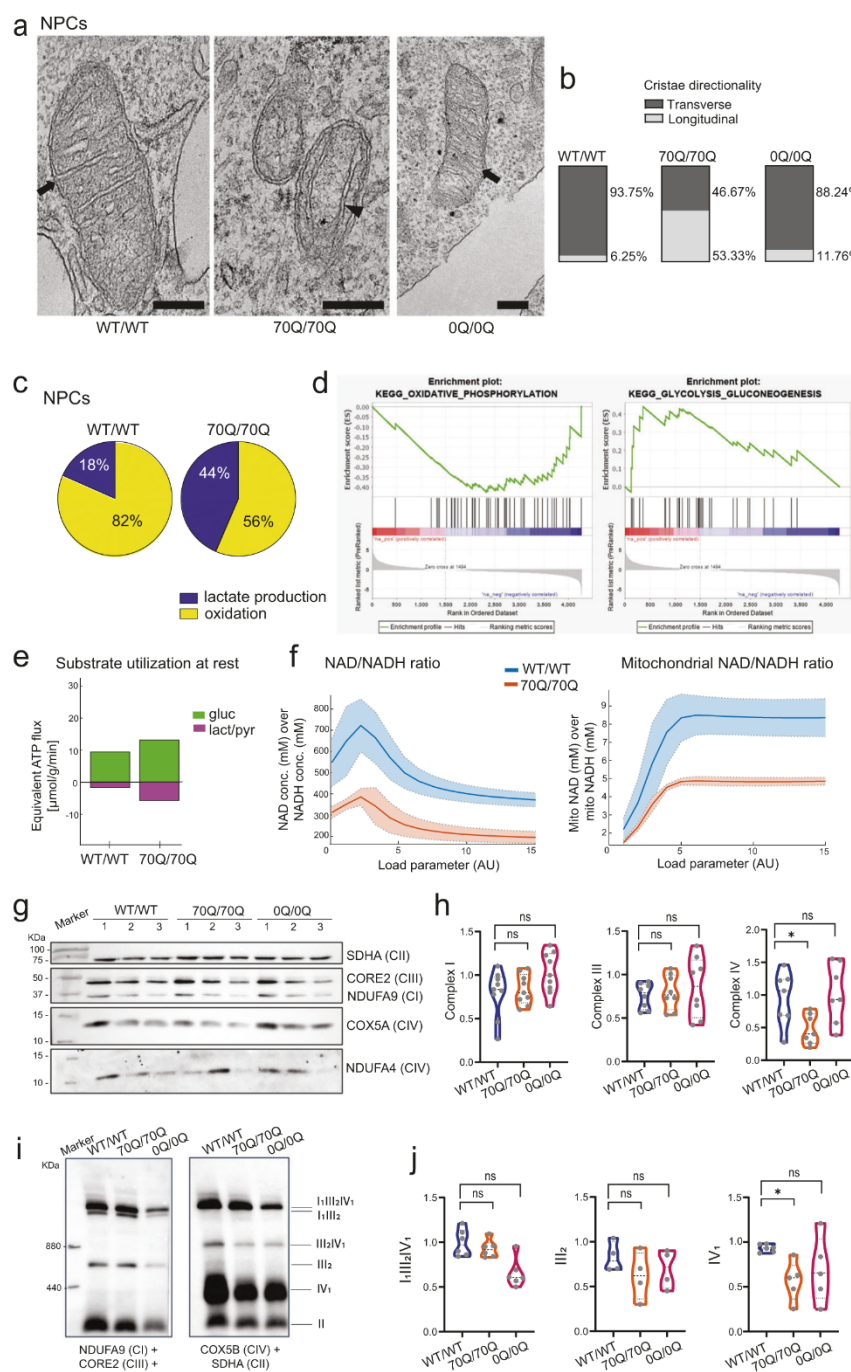
(Supplementary Fig. 8b, c). Next, we performed multi-omics integration. The network of significantly dysregulated transcripts, proteins and metabolites along with predicted intermediate miRNAs and transcription factors arranged in a comet-shaped network (Fig. 7a, Supplementary Fig. 8d). At the core of the network, we found factors related to senescence and Hippo signaling (SP1, SIN3B, YAP1), axon guidance (NRPI, GATA3, NRF1), mISR (ATF4), and the bioenergetic-associated metabolite L-carnitine (Fig. 7a). CHCHD2 and CHCHD10 were closely connected with this core network, indicating that these two proteins have strong interactions with each other within the biological system. Another member of the family, CHCHD1, was also found in close distance (Fig. 7a). Within the network, there were also other bioenergetic metabolites (Acetyl-CoA and lactate) and complex IV factors (COX6C).

qPCR analysis of members of the core network confirmed that Hippo signaling genes SIN3A, SIN3B and YAP1 were downregulated in 70Q/70Q NPCs compared to WT/WT NPCs, showing that engineered cells could recapitulate changes seen in patient-derived cells (Fig. 7b). The same downregulation occurred in OQ/OQ NPCs compared to WT/WT NPCs, which suggested that removal of the CAG/CCG region may not be sufficient to revert these gene expression modifications in NPCs (Fig. 7b).

Overall, the multi-omics integration analysis of HD neurons highlighted the dysregulation of pathways associated with neurodevelopment, axon guidance, Hippo signaling, and carbon metabolism (Fig. 7c, Supplementary Fig. 8e). CHCHD2 was located close to the core of this integrated network, underscoring its relevance in the neuro-pathophysiology of mHTT.

CHCHD2 modulation affects neurite growth and mitochondrial morpho-dynamics

Lastly, we aimed to investigate the impact of manipulating CHCHD2 expression levels in human neural cells. We first assessed the response of homogenous neuronal cultures to the repression of CHCHD2, motivated by data from cerebral organoids indicating that CHCHD2 reduction might be involved in the pathogenesis of HD. HTT is known to play a role in axonal morphogenesis and function^{8,52}, and our omics analysis in neurons indicated the disruption of axon guidance and neurodevelopment-related pathways. Therefore, we focused on the neurite outgrowth capacity of NGN2 neurons using a high-content microscopy-based platform (Fig. 7d)⁵³. We employed a control engineered iPSC line in which we integrated the NGN2 cassette in the safe harbor locus, allowing rapid and robust NGN2 differentiation. Upon transfection with small interfering RNA (siRNA) constructs, we measured the dendritic and axonal outgrowth (Fig. 7d). In agreement with the known role of BDNF in neuronal branching⁵⁴, knock-down of BDNF in NGN2 neurons impaired the development of axonal and dendritic structures (Fig. 7e, Supplementary Fig. 9a). Similar defects in axonal and dendritic branching occurred in NGN2 neurons in which CHCHD2 or HTT were knocked down (Fig. 7e). These defects were stronger than



those observed in the case of VEGFA knock-down, a gene that has also been associated with neurite outgrowth (Supplementary Fig. 9a). The detrimental impact of WT HTT downregulation on neuronal morphogenesis underscores the need for cautious consideration in therapeutic approaches aiming at repressing HTT. In fact, all neural model systems carrying mHTT that we used (NPCs, cerebral organoids, neuruloids, and NGN2 neurons) showed a slight reduction in the

amount of WT HTT levels (Supplementary Fig. 9e–h, j), implying that HD phenotypes are potentially associated with reduced WT HTT.

To determine the consequences of CHCHD2 overexpression in mutant neural cells, we focused on 70Q/70Q NPCs and employed adeno-associated viruses (AAV) encoding for either CHCHD2-GFP or GFP alone. The expression signature of CHCHD2 and TOM20 in 70Q/70Q NPCs did not change upon transduction with GFP-AAV

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Fig. 5 | Neurometabolic defects induced by mHTT. **a** Representative electron microscopy images of mitochondria within WT/WT NPCs, 70Q/70Q NPCs, and OQ/OQ NPCs. Arrows indicate mitochondrial cristae with transverse direction with respect to mitochondrial outer membrane. Arrowhead indicates cristae with longitudinal direction. Data were repeated in two independent experiments. Scale bar: 200 nm. **b** Quantification of cristae direction in NPCs from WT/WT, 70Q/70Q and OQ/OQ. For each sample, a minimum of 15 different mitochondrion were considered out of at least two biological replicates. **c** Proteomic-driven functional metabolic analysis (see Supplementary Data 7) depicting relative glucose utilization at resting energy demands in NPCs. **d** Gene Set Enrichment Analysis (GSEA) showing decreased oxidative phosphorylation (OXPHOS) and increased glycolysis/gluconeogenesis in 70Q/70Q NPCs compared to WT/WT NPCs. **e** Substrate utilization at rest in NPCs from WT/WT and 70Q/70Q. Energetic capacities were

evaluated by computing the changes in metabolic state elicited by an increase of the ATP consumption rate above the resting value. mHTT-carrying cells showed higher consumption of glucose (gluc) and higher production of lactate-pyruvate (lact/pyr). **f** Metabolic state variables for NAD/NADH ratio and mitochondrial NAD/NADH ratio in NPCs in dependence of increasing energetic demands. Lines and colored areas represent mean $\pm$ s.d. **g, h** SDS-PAGE analysis and related quantification of mitochondrial complex I, III, and IV subunits in WT/WT NPCs, 70Q/70Q NPCs, and OQ/OQ NPCs. $n = 3$ independent biological replicates per line run in three different blots; * $p < 0.05$, ns: not significant; unpaired two-tailed Welch t test. **i, j** Blue native PAGE analysis and related quantification of complexes III₂ and IV, and supercomplexes I₁III₂IV₀₋₁ assembly in WT/WT NPCs, 70Q/70Q NPCs, and OQ/OQ NPCs. $n = 3$ independent biological replicates per line run in two different blots; * $p < 0.05$, ns: not significant; unpaired two-tailed Welch t test.

(Supplementary Fig. 9c), and the transduction of either CHCHD2-GFP-AAV or GFP-AAV led to similar levels of GFP signal (Supplementary Fig. 9d). As expected, CHCHD2 levels were significantly higher in 70Q/70Q NPCs transduced with CHCHD2-GFP-AAV compared to those transduced with only GFP-AAV (Fig. 7f, g). By focusing only on the GFP-positive cells, we then quantified the amount of TOM20, and found that CHCHD2 overexpression led to a significant decrease in TOM20 signal (Fig. 7f, g), thus reverting the diseased phenotype that we previously observed in cerebral organoids and NPCs carrying mHTT (Fig. 4g).

In conclusion, repression of CHCHD2 in human neurons adversely affected neurite branching capacity, while its overexpression in neural cells carrying mHTT led to amelioration of mitochondrial morpho-dynamics. These findings highlight the relevance of CHCHD2 in the neuronal and metabolic pathology of HD.

Discussion

Increasing evidence from studies in mice and humans suggests that mutant HTT could impact the physiological development of the brain^{4,5,19}. Our findings based on various human brain organoid differentiation paradigms demonstrate that mHTT disrupts the overall neurodevelopmental process and particularly the organization of neural progenitors. These results align with recent studies in mice and human fetuses showing that mHTT interferes with the cell cycle of apical progenitors, causing reduced proliferation and premature neuronal lineage specification¹⁹.

By exploring mechanisms underlying defective human neurodevelopment caused by mHTT, we identified an early dysregulation of CHCHD2 expression. CHCH domain-containing proteins are imported into the mitochondrion and have been suggested to play a role in neurodegenerative diseases^{32,33}. CHCHD2 contributes to the maintenance of mitochondrial morphology and dynamics, and its loss has been reported to disrupt mitochondrial organization in models of Parkinson's disease^{35,36}. CHCHD2 expression was previously found to promote neural-ectodermal lineage differentiation of human iPSCs⁵⁷, indicating that its presence may be important during neurogenesis. Dysregulation of CHCHD2 has also been observed in neural cells carrying mHTT that were differentiated from human embryonic stem cells (hESCs) and iPSCs^{58,59}. However, the role of CHCHD2 within the pathogenesis of HD remained elusive.

CHCHD2 is known to regulate mitochondrial dynamics and mitochondrial integrated stress response (mISR)²⁶. mISR is a process that rises in stress conditions such as those associated with impaired mitochondrial function³⁵. Accordingly, we identified a mISR signature in human neural cells carrying mHTT that was associated with aberrant mitochondrial morpho-dynamics and specific defects in mitochondrial complex IV assembly. Elimination of the poly-Q/poly-P region reverted the abnormal CHCHD2 expression and the associated mISR signature and mitochondrial defects, including those affecting cristae organization and complex IV assembly. Moreover, CHCHD2 over-expression

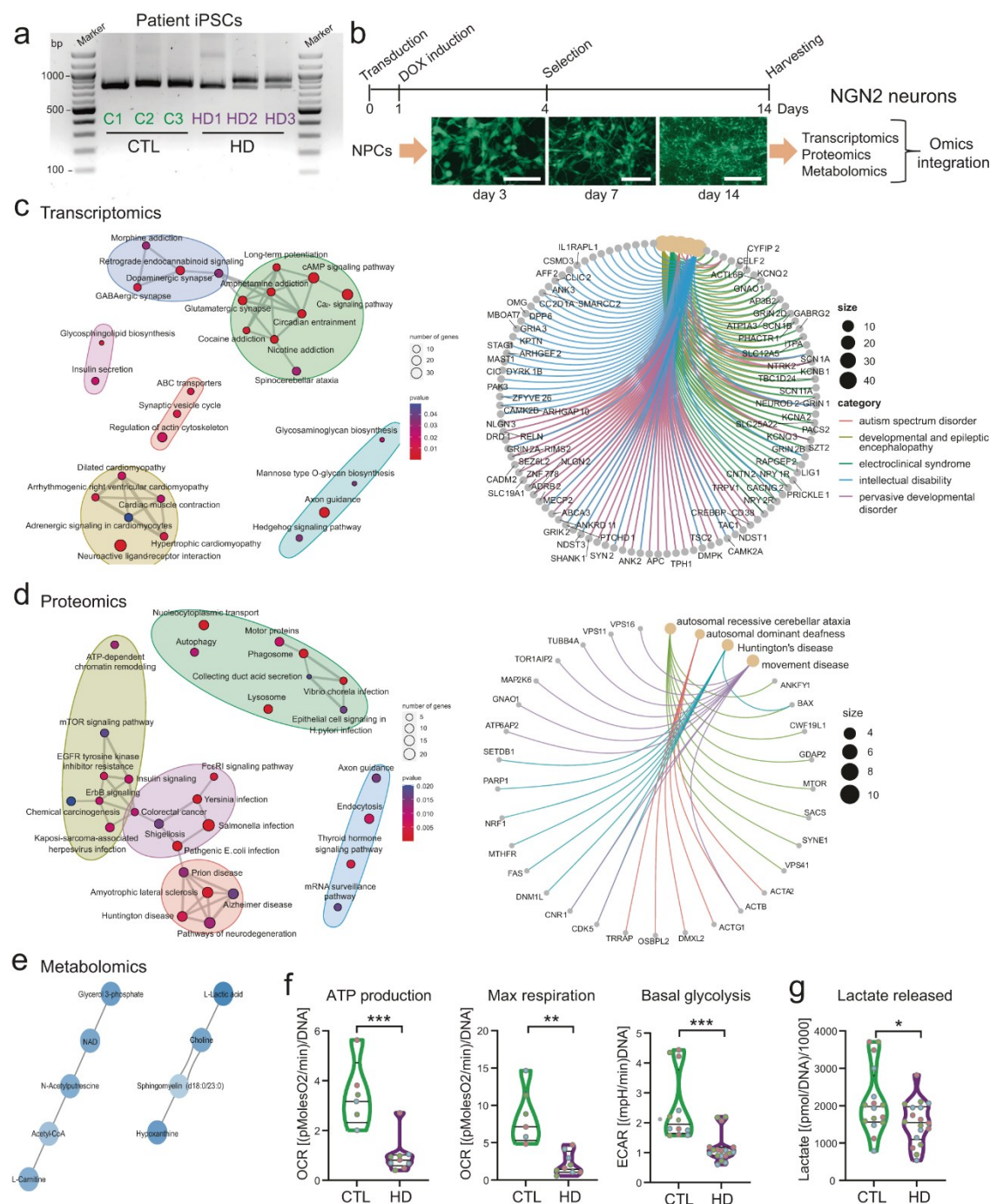
in mutant NPCs ameliorated mitochondrial morpho-dynamics. These findings suggest that mHTT leads to CHCHD2 dysregulation, which in turn impairs mitochondrial function and organization in early neural cells.

Dysfunction of mitochondrial activity and network morphology have been observed in multiple model systems of HD, including iPSC-derived medium spiny neurons^{21,41,42,60–63}. Even Pridopidine, currently being investigated as a treatment for HD in Phase III study (NCT04556656), has been shown to repress mitochondrial stress and network defects⁶⁴. However, again in agreement with a neurodegenerative view of the disease, mitochondrial dysfunction in HD models is usually thought to occur mainly within mature neuronal cells. Our data instead indicate that aberrant CHCHD2 expression and related mitochondrial morpho-function impairment can occur early during neurodevelopment.

Such early mitochondrial dysfunctions is expected to affect the overall process of neurodevelopment⁶⁵. Impairment of the cellular NAD⁺/NADH redox balance caused by metabolic perturbation can in fact lower the global rate of protein synthesis, thereby slowing down the segmentation clock and overall developmental rate⁶⁶. In particular, mitochondrial morphology and metabolism are emerging as important regulators of early neurogenesis^{67–69}. Mitochondrial dynamics within NPCs regulate the pace of development⁷⁰, and help orchestrate the balance between proliferation and neuronal specification⁷¹. A correct metabolic programming towards OXPHOS starts to develop at the level of NPCs^{46,47,72,73}. In our models, we found that both undifferentiated iPSCs and NPCs were able to respond to OXPHOS impairment by upregulating glycolytic metabolism. However, impaired NAD⁺/NADH ratio became evident only in NPCs. Differentiated neurons could not compensate these defects and developed cell autonomous impairment of both OXPHOS and glycolysis. The establishment of neural fate might then pose greater pressure on cells that start relying more on OXPHOS, ultimately leading to metabolic stress and functional impairment. Hence, mHTT may interfere with the physiological metabolic programming that is required for enabling neural fate commitment.

Despite a disruption in neural progenitors, the generation of neurons (either appearing physiologically within brain organoids or forced through NGN2 overexpression) still occurred in the presence of mHTT. Indeed, we observed increased upregulation of genes related to neurodevelopment in mutant neural progenitor cultures forced to be converted into neurons. This is in agreement with premature neuronal generation observed in human fetuses carrying mHTT¹⁹. However, even if neurons can be generated, their functionality and resilience may be compromised. Cells carrying mHTT that prematurely commit to neural differentiation could thus produce neurons that are more susceptible to degeneration upon stress. Therefore, the loss of mechanisms protecting from stress and decompensation may play a role in the development of HD. Here, we found that CHCHD2 reduction in HD patient-derived neurons impaired neuronal branching capacity

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and its overexpression in mutant NPCs promoted a healthier mitochondrial state. In accordance, recent findings point towards a protective role for CHCHD2 up-regulation in HD as a protection response against oxidative stress⁴.

These results are important for our understanding of the pathomechanisms of HD, as the prevalent view of the disease is that it develops as a degeneration of mature neurons⁷⁵. If brain defects

caused by mHTT instead develop early in life, therapeutic strategies should be administered during development. It is thus possible that therapeutics based on lowering mHTT, as performed in the phase III clinical trial (NCT03761849) developed by Roche⁷⁶, might not be as effective as hoped for. If such administration is given during adult life, the brain circuitry has already been altered and may not be effectively repaired anymore. Accordingly, we found that defects in neural

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Fig. 6 | Multi-omics signature of NGN2 neurons from individuals with HD underscores developmental and metabolic defects. **a** PCR analysis of HTT in NPCs derived from three healthy controls (C1, C2, C3) and three individuals with HD (HD1, HD2, HD3, carrying WT/180Q, WT/58Q, and WT/44Q, respectively). Data were repeated in three independent experiments. **b** Schematics of Neurogenin 2 (NGN2)-based neuronal induction in NPCs from healthy controls (C1, C2, C3) and HD individuals (HD1, HD2, HD3). Scale bar: 100 μ m. **c** Enrichment analysis of differentially expressed genes (DEGs) in NGN2 neurons from HD individuals compared to controls, hypergeometric test one-sided with FDR adjustment for multiple comparisons (filter for p value < 0.05). Left: over-representation analysis (ORA) of upregulated DEGs for KEGG pathways; right: ORA of upregulated DEGs for human diseases. Yellow nodes represent the enriched diseases and gray nodes their associated genes, with colored edges indicating their connection (see Supplementary Data 7). **d** Enrichment analysis of differentially expressed proteins (DEPs)

in NGN2 neurons from HD individuals compared to controls, hypergeometric test one-sided with FDR adjustment for multiple comparisons (filter for p -value < 0.05). Left: ORA of upregulated DEPs for KEGG pathways; right: ORA of upregulated DEPs for human diseases (see Supplementary Data 8, 9). **e** Enrichment analysis of differentially expressed metabolites (DEMs) in NGN2 neurons from HD individuals compared to controls (see Supplementary Data 10). Interactions between the significantly dysregulated metabolites. **f** Mitochondrial bioenergetics based on oxygen consumption rate (OCR) and glycolysis based on extracellular acidification rate (ECAR) measured by Seahorse profiling in NGN2 neurons from controls (C1, C2, C3) and HD individuals (HD1, HD2, HD3). $n = 3$ independent biological replicates per line (different colors of dots refer to the three replicates). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; unpaired two-tailed t test. **g** Quantification of lactate released in the supernatant by NGN2 neurons at the end of Seahorse experiments. * $p < 0.05$; unpaired two-tailed t test.

progenitors and brain organoids occurred at a time point where toxic mHTT aggregates have not yet developed. Smaller intracranial volumes can be observed early in pre-manifest HD mutation carriers^{14–16}. These findings raise the possibility that HD pathogenesis may also involve a loss of function mechanism, caused by the reduction of WT HTT. Indeed, WT HTT has been proposed to play a role in neurodevelopment, where it could regulate mitotic spindle orientation thereby determining cortical progenitor fate¹², and participate in axonal transport modulation⁷⁷. In agreement with this, we found that downregulation of WT HTT was sufficient to impair neuronal branching capacity.

Individuals with HD often suffer from weight loss and cachexia irrespective of food intake^{78–80}. In fact, the metabolic network impairment has been suggested as a progression biomarker of pre-manifest HD⁸¹. We found that mHTT caused a metabolic state indicative of elevated energy consumption at rest. This feature, also known as hypermetabolism, leads to weight loss, and is associated with greater functional decline in cancer patients or individuals with amyotrophic lateral sclerosis⁸². Hypermetabolism has recently been found to occur in the presence of OXPHOS-impairing mutations that can lead to mISR activation³⁵. Previously, we and others have demonstrated that OXPHOS-impairing mutations cause aberrant neuronal morphogenesis and defective brain organoid development^{83,84}. Hence, it is possible that CHCHD2 could represent a target for early intervention in HD, as its modulation would impact mitochondrial morpho-function, which in turn could be important for morphogenesis and neurodevelopment on the one hand and for overall systemic metabolic resilience on the other.

Our integrative multi-omics analysis of HD patient-derived neurons indicated that CHCHD2 belonged to a core dysregulated network composed of genes/proteins/metabolites associated with neurodevelopment, axon guidance, Hippo signaling, carbon metabolism, and mISR. Among these factors, SIN3A and YAP1 may be particularly interesting, given that SIN3A function counteracted neurodegeneration in a drosophila model of HD⁸⁵, and YAP1 was found dysregulated in HD mice and in post-mortem patient brain⁷⁹. In fact, YAP activators ameliorated symptoms and disease phenotypes in different HD models^{86,87}, and modulation of YAP activity rescued key HD-related neurodevelopmental phenotypes in neurospheres carrying mHTT⁸⁸. Hence, by targeting CHCHD2 expression, other associated factors may possibly also be positively influenced. Future studies should address whether over-expression of these factors might lead to ameliorate the neuronal defects caused by mHTT. It was previously found that polyQ inclusion sequester RNA molecules in an overexpression model of polyQ-ATXN1⁸⁹. It is tempting to speculate that something similar might also be occurring in other polyQ diseases such as HD, where polyQ inclusions could sequester RNAs or transcription factors causing their impairment.

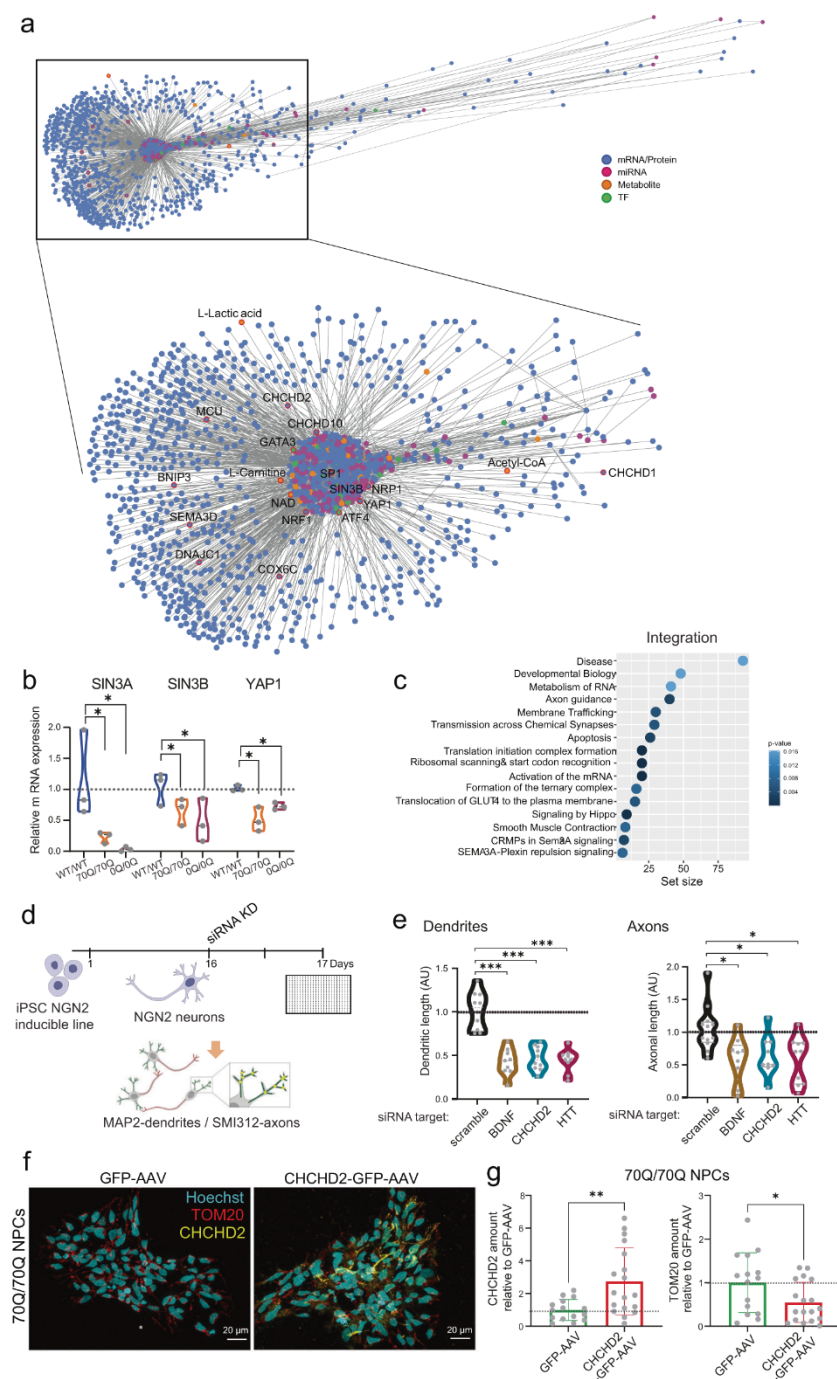
It is important to note that our study primarily examined cells carrying mHTT on both alleles, a condition in which potential compensatory effects of WT HTT are absent. Hence, additional research would be required to further dissect the role in CHCHD2 in different HTT genotypes, given that homozygous and heterozygous forms are not clearly different in HD patients^{90,91}. At the same time, it was reassuring to see that NGN2 neurons from HD patients carrying WT/180Q, WT/58Q, and WT/44Q recapitulated similar defects regardless of the poly-Q length. Indeed, a recent study using cerebral organoids obtained from hESCs carrying WT/45Q, WT/65Q and WT/81Q described similar features indicative of neurodevelopment impairment irrespective of the polyQ length and the homozygous or heterozygous state²⁴. The consequences of CAG/CCG elimination also need to be examined in more details. 0Q/0Q cells showed rescue of mitochondrial phenotypes but the expression for genes associated with Hippo signaling was not ameliorated. Furthermore, 0Q/0Q NPCs exhibited changes in mitochondrial ultrastructure that were not seen in 70Q/70Q NPCs. The biological meaning of these changes remains unclear. Further investigations are also warranted to address the impact of somatic deletion of the polyQ region directly in neural cells, in contrast to applied as in-frame deletion in iPSCs. Lastly, as CHCHD2 belongs to the high-confidence interactome map of neurodegenerative-associated human proteins⁹², it will be important to be determined whether it could represent a direct binding partner of mHTT.

Methods

Human iPSC lines

Our research complies with all relevant ethical regulations. Ethical approval for using iPSCs from HD individuals and control subjects was obtained from the Ethic Committee of the University Clinic Düsseldorf (study number 2019-681 approved on October 11, 2019).

WT/WT iPSCs for genome editing were derived using Sendai viruses from a healthy middle-aged male individual (BIHi050-A/SCV1113). iPSCs from three male individuals with HD were described before: HD1 with WT/180Q (BIHi035-A)⁴⁹, HD2 with WT/58Q (BIHi288-A)⁵⁰, and HD3 with WT/44Q (BIHi033-A)⁵⁰. Control male iPSCs used for comparison with HD male iPSCs were derived before: C1 (TFBJ, HHUUKDi009-A)⁴⁶, C2 (XM001, BIHi043-A)⁹³, and C3 (BIHi005-A). For engineering an inducible NGN2 iPSC line, we used the control healthy male iPSC line C3 (BIHi005-A). All iPSCs were cultured feeder-free on Matrigel-coated (Corning, USA) 6-well plates (Greiner Bio-One, Austria) in iPSC-Brew consisting of StemMACS iPSC-Brew XF basal medium supplemented with 1:50 StemMACS iPSC-Brew XF supplement (both Miltenyi Biotec, Germany) and 1:500 MycoZap-Plus-CL (Lonza, Switzerland). iPSC cultures were kept in a humidified atmosphere of 5% CO₂ and 5% oxygen at 37 °C. We routinely monitored all cultures against mycoplasma contamination using PCR. Cells were passaged at



70–80% confluence with 0.5 μM EDTA (Invitrogen, USA) in 1xPBS (Gibco, USA). 10 μM Rock inhibitor (Enzo Biochem, USA) was added after splitting to promote survival.

Engineering huntingtin in human iPSCs

Isogenic clones with a homozygous (HTT WT/70Q) and heterozygous (HTT 70Q/70Q) allelic combinations carrying 70Q insert were

engineered from control (WT/WT) BIH-SCVII13 iPSC line with healthy background. We used clustered regularly interspaced short palindromic repeats (CRISPR) and high-fidelity Cas9 (eCas9), which has limited off-target effects²⁷. We used CRISPR/Cas9 induced homology directed repair (HDR) with 2354 nts dsDNA donor template encoding patient reference sequence carrying 70Q consisting of both CAG and CAA triplets including 3' and 5' homology arms. Constructs were

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Fig. 7 | CHCHD2 belongs to the multi-omics network of HD neurons and its manipulation affects neurite growth and mitochondrial morpho-dynamics. **a** 3D multi-omics network. Significantly dysregulated mRNAs, proteins, and metabolites along with their predicted miRNAs and transcription factors (TFs) are arranged in a comet-shaped network. CHCHD2 and CHCHD10 are located closely to the core of the network. **b** qPCR analysis of components of the core network related to Hippo signaling in NPCs. Expression level of each gene was related to that of housekeeping genes ACTB and OAZ1. Mean $\pm$ s.e.m.; $n = 3$ independent biological replicates (dots) per line; $^*p < 0.05$; unpaired two-tailed Welch t test. **c** Enrichment analysis for Reactome biological pathways affected by the dysregulated mRNAs/proteins of the multi-omics network; hypergeometric test one-sided with FDR adjustment for multiple comparisons (filter for adj. p value ≤ 0.05). **d** Schematics

of small interfering RNA (siRNA)-mediated knockdown (KD) in NGN2 neurons from NGN2-inducible control iPSCs. Quantification of neuronal arborization assessed by high-content imaging based on antibodies labeling axons (SMI32) and dendrites (MAP2). **e** Quantification of branching outgrowth in NGN2 neurons following siRNA KD. Dot indicates different replicates out of three independent experiments. $^*p < 0.05$, $^{***}p < 0.001$ compared to scramble siRNA KD; Kruskal-Wallis test with Dunn's multiple comparisons test. **f** Representative images of 70Q/70Q NPCs transduced with either GFP-AAV or CHCHD2-GFP-AAV. Scale bar: 20 μ m. **g** Quantification of CHCHD2 and TOM20 amount in GFP-positive 70Q/70Q NPCs transduced with either GFP-AAV or CHCHD2-GFP-AAV. Data shown as mean $\pm$ s.d. Dots represent individual images collected from two independent experiments. represent $^{**}p < 0.01$, $^*p < 0.05$; unpaired two-tailed Welch t test.

commercially synthesized as a synthetic HTT donor construct and assembled from synthetic oligonucleotides by GeneArt Gene Synthesis service (Thermo Fisher Scientific, USA). Silent mutations within single guide RNAs (sgRNAs) were introduced in proximity to each of the two sgRNA protospacer adjacent motif (PAM) sites (3 and 6 nt downstream) to prevent recurrent Cas9 cutting in edited cells. Synthesized template was inserted into pMK-RQ (kanR)/pMA-RQ (ampR) plasmid, purified from transformed bacteria, determined by spectroscopy and verified by sequencing. sgRNAs (sgHTT#1, sgHTT#2) targeting upstream and downstream of the HTT polyQ region were designed using CRISPOR (Supplementary Fig. 1a, Supplementary Data 11). The oligomer pairs were annealed and cloned separately into two pU6-CAG-eCas9-Venus plasmids carrying eSpCas9 variant from eSp-Cas9(L1) plasmid (Addgene ID 71814) to reduce off-target effects and improve on-target cleavage²⁷. To upregulate homology directed repair (HDR) of eCas9 induced double-strand breaks with dsDNA donor template, we applied ectopic expression of BRCA1 and BCL-XL. To downregulate the non-homologous end joining (NHEJ) pathway we applied dominant-negative sub-fragment of 53BP1 (dn53BP1), which counteracts endogenous 53BP1²⁸. Plasmids encoding components of the DNA repair pathways (human BRCA1, BCL-XL and mouse dn53BP1) were kindly obtained from Bruna Paulsen (dn53BP1) and Xiao-Bing Zhang (BCL-XL). For generation of dn53BP1, a fragment containing the tudor domain (residues 1,221 to 1,718 of mouse 53BP1) was amplified and sub-cloned into a CAG expression plasmid and sequenced. For the generation of BCL-XL, a fragment containing the BCL-XL was amplified and sub-cloned into a pEF1-BFP expression plasmid and sequenced. pU6-sgHTT#1-CAG-eCas9-Venus and pU6-sgHTT#2-CAG-eCas9-Venus plasmids targeting upstream and downstream of the CAG/CCG stretches of the HTT gene, plasmids encoding 70Q HDR template and BRCA1, BCL-XL and mouse dn53BP1 were transformed into super competent DHA bacteria strain using heat shock and cloned. Plasmid DNA was extracted using the PureYield plasmid miniprep system (Promega) and transfected into the control iPSC line. In addition to the BIH-SCVII13 iPSCs with introduced 70Q templates, we also generated clones harboring HTT alleles using a strategy based on NHEJ mediated excision of the poly Q/P repeats region leading to in-frame reannealing of the DSB. By transfection of pU6-sgHTT#1-CAG-eCas9-Venus and pU6-sgHTT#2-CAG-eCas9-Venus with BCL-XL plasmids, we generated DSBs which upon re-ligation with the lack of HDR template resulted into an in-frame HTT coding region, lacking the N-terminal Q/P repeats. Transient transfection of plasmids was carried out in BIH-SCVII13 iPSC line grown in feeder-free conditions in StemMACS™ iPS-Brew XF culture media (Miltenyi Biotec, Germany) in a 6-well cell culture plate. One day prior to transfection, we dissociated the cells using Accutase (Sigma-Aldrich, USA) and seeded $\sim 1 \times 10^5$ cells per well of a pre-coated 6-well plate as single cells or small clumps. Cells were cultivated in fresh medium containing 10 mM Rock inhibitor (Enzo Biochem, USA) overnight. Transfection was performed using Lipofectamine™ 3000 Transfection Reagent (Thermo Fisher Scientific, USA), according to the manufacturer's protocol. The plasmids were

diluted up to 2 mg DNA in 125 ml of Opti-MEM reduced serum medium and added as the DNA-lipid complex to one well of a 6-well plate in a dropwise manner with addition of 5 mM Rock inhibitor to the culture medium for 24 h. Medium was changed on the following day and the cells were kept 48 h in culture until fluorescence-activated cell sorting (FACS). Dissociated cells using Accutase for 5 min were washed and resuspended with PBS. Then, cells were filtered using Falcon polystyrene test tubes (Corning, USA) and transferred to new Falcon polypropylene test tubes. Two colors FACS enrichment was performed using BD FACSARIA III at the MDC FACS Facility for cells expressing high levels of eCas9-sgRNAs, and BCL-XL (Venus, BFP). Sorted cells were suspended in recovery mTeSR™ medium (StemCell Technologies, Canada) with 1X Penicillin-Streptomycin (P/S) (Gemini Bio-products) and ROCK inhibitor and plated in low concentrations onto 6-well plates for the establishment of single cell derived colonies (5000 cells/well). Growing single cell-derived colonies were transferred from 6-well plates to one well each of 24-well plate and maintained until the colony grew big enough to be partially harvested for DNA isolation using Phire Animal Tissue Direct PCR Kit (Thermo Fisher Scientific, USA) according to manufacturer's protocol. PCR reaction was carried out using 10 ng gDNA in 25 ml with chemical hot-Start AmpliTaq Gold DNA Polymerase (Thermo Fisher Scientific, USA) with an annealing temperature of 55 °C and GC enhancer reagent (Thermo Fisher Scientific, USA). For Sanger sequencing, the PCR products were gel purified using the Wizard SV Gel and PCR Clean-Up System (Promega) and cloned into pJET cloning vectors using the CloneJET PCR Cloning Kit (Thermo Fisher Scientific, USA). Cloned PCR products were submitted to LGC for Sanger sequencing. Karyotype analysis was performed by MDC Stem Cell Core Facility. Briefly, DNA was isolated using the DNeasy blood and tissue kit (Qiagen, USA). SNP karyotyping was assessed using the Infinium OmniExpressExome-8 Kit and the iScan system from Illumina. CNV and SNP visualization was performed using KaryoStudio (v. 1.4) (Illumina, USA). Primer sequences, gRNA sequences, and HDR sequences are reported in Supplementary Data 11.

Derivation of neural progenitor cells (NPCs)

To generate neural progenitor cells (NPCs) from iPSCs, we applied our published protocol⁹⁴. Briefly, iPSCs were detached from Matrigel-coated plates using Accutase (Sigma-Aldrich, USA) and the collected sedimented cells were transferred into low-attachment petri dishes and kept for two days in M1 medium (1x KnockOut DMEM, 1x KnockOut serum, 0.1 mg/ml PenStrep, 2 mM Glutamine, 1x NEAA, 1 mM Pyruvate [all Gibco, USA], 1x MycoZap-Plus-CL [Lonza, Switzerland], 3 μ M CHIR 99021 [Caymen Chemical, USA], 10 μ M SB-431542 [Caymen Chemical, USA], 1 μ M Dorsomorphin [Sigma-Aldrich, USA], 500 nM Purmorphamine [Miltenyi Biotec, Germany]). From day 2 to day 6, the media was switched to M2 medium (50% DMEM-F12, 50% Neurobasal, 0.5x N-2 supplement, 0.5x B-27 supplement without Vitamin A, 0.1 mg/ml PenStrep, 2 mM Glutamine [all Gibco, USA], 1x MycoZap-Plus-CL [Lonza, Switzerland], 3 μ M CHIR 99021 [Caymen Chemical, USA], 10 μ M SB-431542 [Caymen Chemical, USA],

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1 μ M Dorsomorphin [Sigma-Aldrich, USA], 500 nM Purmorphamine [Miltenyi Biotec, Germany]). On day 6, the suspended cells were transferred onto Matrigel-coated (Corning, USA) 6-well plates (Greiner Bio-One, Austria) using sm+ medium (50% DMEM-F12, 50% Neurobasal, 0.5x N-2 supplement, 0.5x B-27 supplement without Vitamin A, 0.1 mg/ml PenStrep, 2 mM Glutamine [all Gibco, USA], 1x MycoZap-Plus-CL [Lonza, Switzerland], 3 μ M CHIR 99021 [Cayman Chemical, USA], 500 nM Purmorphamine [Miltenyi Biotec, Germany], 150 μ M ascorbic acid [Sigma-Aldrich, USA]). NPCs were maintained in this media without rock inhibitor and used for experiments between passage 7 and 20.

Generation of unguided cerebral organoids

Unguided cerebral organoids were generated from WT/WT and 70Q/70Q iPSCs using a modified version of a published protocol⁹⁵. At day 0, iPSCs from one 80% confluent well were washed with 1x PBS (Gibco, USA) and treated with Accutase (Sigma-Aldrich, USA) for 3 min at 37 °C. Subsequently, cells were collected in 5 ml iPS-Brew and spun down for 5 min at 270 $\times$ g. After removing the supernatant, cells were resuspended in 1 ml embryoid body (EB) media (1x DMEM-F12, 1:5 KnockOut-Serum, 1x NEAA, 0.1 mg/ml PenStrep, 1x GlutaMAX [all Gibco, USA], 7 nM 2-mercaptoethanol [Merck, Germany], 4 ng/ml bFGF [PeproTech, USA], 10 μ M Rock inhibitor [Enzo Biochem, USA]). Cells were counted manually, and 9000 cells were plated in 150 μ l EB media in each well of a round bottom, ultra-low attachment 96-well plate (Corning, USA). Next, the plate was spun down at 500 rpm for 2 min to assure the aggregation of cells in the center of the wells. On day 2 and day 4, 50% of the EB media was replaced with EB media without ROCK inhibitor and bFGF. At day 5, each EB was transferred to a well of a 24-well plate (Greiner bio-one, USA) in 250 μ l neural induction media (IM) (1x Neurobasal, 1x N-2 supplement, 1x NEAA, 1x GlutaMax, 0.1 mg/ml PenStrep [all Gibco, USA], 1 μ g/ml heparin [Sigma-Aldrich, USA]). On day 6 and 10, another 250 μ l IM was added to each well. At day 11, organoids were embedded in droplets of 30 μ l Matrigel (Corning, USA)⁹⁵, and 16 organoids each were transferred to 60 $\times$ 15 mm cell culture dishes (Greiner bio-one, USA) and cultured for 48 h in 5 ml IM at 37 °C with 5% CO₂. After this, IM was replaced with neuronal differentiation media 1 (DMI) (50% DMEM-F12, 50% Neurobasal, 0.5x N-2 supplement, 2x B-27 supplement without vitamin A, 0.1 mg/ml PenStrep, 1x GlutaMax, 0.5x NEAA [all Gibco, USA], 0.35 nM 2-mercaptoethanol [Merck, Germany], 2.5 μ M insulin [Sigma-Aldrich, USA], 3 μ M CHIR99021 [Cayman Chemical, Germany]). After 2 days, the media was replaced by fresh DMI and at day 17, the organoids were placed on an orbital shaker (set to 85 rpm) and the media was replaced by DM2 (50% DMEM-F12, 50% Neurobasal, 0.5x N-2 supplement, 1x B-27 supplement, 0.1 mg/ml PenStrep, 1x GlutaMax, 0.5x NEAA, 1:100 chemically defined lipid concentrate [all Gibco, USA], 0.35 nM, 2-mercaptoethanol [Merck, Germany], 2.5 μ M insulin [Sigma-Aldrich, USA], 0.4 μ M ascorbic acid [Sigma-Aldrich, USA], 12 mM HEPES solution [Biochrom, Germany]). From this point on, full media replacements took place every 3-4 days. At day 40, the media was changed to maturation media (MM) (1x Neurobasal, 1x B-27 supplement, 0.1 mg/ml PenStrep [all Gibco, USA], 0.35 nM 2-mercaptoethanol [Merck, Germany], 0.4 μ M ascorbic acid [Sigma-Aldrich, USA], 20 ng/ml BDNF [Miltenyi Biotec, Germany], 20 ng/ml GDNF [Miltenyi Biotec, Germany], 0.5 mM cAMP [Sigma-Aldrich, USA], 12 mM HEPES solution [Biochrom, Germany]). Media was checked for mycoplasma routinely. Organoid size was measured using ImageJ (v. 1.53a).

Generation of guided region-specific brain organoids

Cortical organoids were generated following a protocol previously published⁹⁶ with minor modifications. On day 0, iPSCs at 80% confluence were incubated with Accutase (Sigma-Aldrich, USA) at 37 °C for 7 min and dissociated into single cells. For aggregation into spheroids, approximately 3×10^6 single cells were seeded per AggreWell-800 well (StemCell Technologies, Canada) in StemMACS iPS Brew XF (Miltenyi Biotec, Germany) supplemented with 10 μ M ROCK inhibitor (Enzo Biochem, USA), centrifuged at 100 $\times$ g for 3 min, and then incubated at 37 °C in 5 % CO₂. After 24 h, spheroids consisting of approximately 10,000 cells were collected from each microwell by gently pipetting the medium up and down with a cut P1000 pipet tip and transferred into a petri dish coated with Anti-adherence solution (StemCell Technologies, Canada). From day 1-6, ES Medium (KnockOut DMEM, 20% Knockout Serum Replacement, 1x NEAA, 1x Sodium Pyruvate, 1x GlutaMAX [all Gibco, USA], 1x MycoZap-Plus-CL [Lonza, Switzerland], 2.5 μ M dorsomorphin [Sigma-Aldrich, USA], 10 μ M SB-431542 [Cayman Chemical, USA]) was changed daily. From day 7-22, neural medium (Neurobasal-A, B-27 Supplement without Vitamin A, 1x GlutaMax [all Gibco, USA], 1x MycoZap-Plus-CL [Lonza, Switzerland]) supplemented with 20 ng/ml EGF (R&D Systems, USA), and 20 ng/ml FGF2 (R&D Systems, USA). From day 22, the neural medium was supplemented with 20 ng/ml BDNF (Miltenyi Biotec, Germany), 20 ng/ml NT-3 (PeproTech, USA), 200 μ M ascorbic acid (Sigma-Aldrich, USA), 50 μ M db cAMP (StemCell Technologies, Canada) and 10 μ M DHA (MilliporeSigma, USA). From day 46, only neural medium containing B-27 Plus Supplement (Gibco, USA) was used for media changes every 2-3 days.

NPC-derived midbrain organoids were generated following a protocol previously published⁹⁰ with some modifications. On day 0, we detached NPCs using Accutase (Sigma-Aldrich, USA) to obtain a single-cell suspension. Using an automatic cell counter (Cytosmart, Netherlands) we gated the cell size 5-19 μ m and seeded 9000 cells per well onto a low-attachment U-bottom 96-well plate (FaCellitate, Germany) in 150 μ l of basal media (50% DMEM-F12, 50% Neurobasal, 0.5x N2 supplement, 0.5x B-27 supplement without Vitamin A, 1x GlutaMAX [all Gibco, USA], 1x MycoZap-Plus-CL [Lonza, Switzerland]) supplemented with 0.5 μ M PMA (Miltenyi Biotec, Germany), 3 μ M CHIR 99021 (Sigma-Aldrich, USA), and 100 μ M ascorbic acid (Sigma-Aldrich, USA). After 2 days, we started ventral patterning for 4 days (in two feedings) by supplementing the basal media with 100 μ M ascorbic acid, 1 μ M PMA, 1 ng/ml BDNF (Miltenyi Biotec, Germany) and 1 ng/ml GDNF (R&D Systems, USA). On day 6 we switched to maturation media by supplementing the basal media with 100 μ M ascorbic acid, 2 ng/ml BDNF, 2 ng/ml GDNF, 1 ng/ml TGF- β 3 (StemCell Technologies, Canada), 100 μ M db cAMP (StemCell Technologies, Canada) and the addition of 5 ng/ml Activin A (StemCell Technologies, Canada). From day 6 on, we refreshed the maturation media (without Activin A, which is exclusively added on day 6) 3 times a week. To measure organoid size, we first took pictures of the organoids every other day with a Nikon Eclipse Ts2 inverted routine microscope using a 4x objective. We measured the area and perimeter from at least 5 organoids per cell line for each time point. We analyzed the images with ImageJ (v. 1.53a) by manually drawing a circle around the organoid⁸³.

Engineering of inducible NGN2 iPSCs

We used the healthy iPSC line C3 (BIHi005-A) to engineer a doxycycline inducible Neurogenin 2 (NGN2) expression cassette to obtain the line BIHi005-A-24. To generate this cell line, we used the strategy of TALEN based gene-editing. Each AAVS1 locus was changed differently; on one AAVS1 allele we inserted the constitutive expression cassette of the reverse trans activator domain (m2rtTA) and on the other one the mNgn2-P2A-GFP-T2A-Puromycine cDNA sequence under the tetracycline responsive element (TRE). To generate the AAVS1-TRE-mNgn2-P2A-GFP-T2A-Puromycine donor plasmid, we amplified the TRE-

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mNgn2-P2A-GFP-T2A-Puromycine sequence from LV-NEP (YS-TetO-FUW-Ng2-P2A-EGFP-T2A-Puro) plasmid from Dr. Thomas Sudhof lab⁵¹, and cloned it into the plasmid AAVSI-iCAG-copGFP (Addgene 66577). To make the lines inducible for doxycycline, the reverse TET trans activator (m2rtTA) was inserted on the other allele of the AAVSI locus using the m2rtTA plasmid containing a Neomycin resistance gene (addgene 60843). The TALEN plasmids to target AAVSI locus were used from addgene: hAAVSI-TAL-L (35431) and hAAVSI-TAL-R (35432). BIHI005-A iPSC cells were transfected with TALEN plasmids targeting AAVSI locus and donor plasmids (AAVSI-TRE-mNgn2-P2A-GFP-T2A-Puro and AAVSI-NEO-M2rtTA). Then we selected the clones which were resistant for both puromycin and neomycin antibiotics. Next, we derived single-cell clones as described²⁷, and selected the clones that express GFP and differentiated to neurons upon exposure to 3 µg/ml doxycycline. The selected clones showed typical pluripotent stem cell morphology, expressed pluripotent markers, and showed normal karyotype.

Derivation of NGN2 neurons

For generation of NGN2 neurons from the inducible NGN2 iPSC line BIHI005-A-24, 3×10^5 iPSCs per well were seeded in a Geltrex (Gibco, USA) coated 6-well plate (Greiner Bio-One, Austria) using StemMACS iPS-Brew medium supplemented with 10 µM Rock inhibitor (Enzo Biochem, USA). On day 0 and day 1, we added induction medium (DMEM-F12, 1x N2 supplement, 1x NEAA, 1 mg/ml Pen/Strep [all Gibco, USA], 10 ng/ml human NT-3 [PeproTech, USA], 10 ng/ml human BDNF [Miltenyi Biotec, Germany], 0.2 µg/ml murine laminin [Sigma-Aldrich, USA]) freshly supplemented with 3 µg/ml doxycycline (Sigma-Aldrich, USA). On day 2, medium was changed to neuronal medium (Neurobasal, 1x B-27 supplement, 1x GlutaMAX, 1 mg/ml Pen/Strep [all Gibco, USA], 1x MycoZapTM Plus-CL [Lonza, Switzerland], 10 ng/ml human NT-3 [PeproTech, USA], 10 ng/ml human BDNF [Miltenyi Biotec, Germany], 0.2 µg/ml murine laminin [Sigma-Aldrich, USA]) freshly supplemented with 3 µg/ml doxycycline (Sigma-Aldrich, USA). Medium was exchanged daily until day 4. From day 6 on, half of the medium was replaced every other day with neuronal medium freshly supplemented with 3 µg/ml doxycycline (Sigma-Aldrich, USA) and 2 µM AraC (Sigma-Aldrich, USA) until the cells were harvested for subsequent experiments.

For NGN2 neurons derived from healthy iPSCs and HD iPSCs, we employed NPCs derived from control iPSCs (C1, C2, C3) and from HD patient-derived iPSCs (H1, H2, H3). The protocol was modified from a published protocol³⁹. For NGN2 virus production, HEK 293 cells were seeded at 70% confluency in DMEM medium in a 150 cm² dish. After cell attachment, the medium was replaced and supplemented with 25 µM Chloroquine and the cells were transfected according to the manufacturer's instructions using Lipofectamine 2000 (Thermo Fisher Scientific, USA) and the following plasmid mix: 8.1 µg pMD2.G, 12.2 µg pMDLg-pRRE, 5.4 µg pRSV-Rev, 5.4 µg FUW-M2-rtTA, 14.3 µg TetO-FUW-NGN2, 14.3 µg TetO-FUW-EGFP. After 24 h, the medium was replaced with DMEM-F12 (Gibco, USA) and 10 µM sodium butyrate (Sigma-Aldrich, USA). Virus-containing supernatant was collected 24 h and 48 h later. To concentrate the virus particles, the supernatant was first centrifuged at $500 \times g$ for 10 min to remove cells and debris and then mixed with 1 volume of cold Lenti-X concentrator (Takara Bio, Japan) to every 3 volumes of lentivirus-containing supernatant and kept overnight at 4 °C. Afterwards, the mixture was centrifuged at $1500 \times g$ for 45 min at 4 °C, and the resulting pellet was resuspended in PBS (Gibco, USA), 1:100 of the original volume. Virus aliquots were stored at -80 °C until further use. For NGN2 neuron generation and culture, 2.5×10^6 NPCs per well were seeded on a Matrigel-coated (Corning, USA) 6-well plate (Greiner Bio-One, USA) using sm+ medium (see NPC generation for media composition). After cells attached (2–24 h), the medium was replaced and supplemented with 4 µg/ml polybrene and 2.25×10^6 transducing units of both EGFP and NGN2

lentivirus. On the next day (day 0), the cells were washed 3 times with PBS and 3 volumes sm+ medium mixed with 1 volume NGN2 medium (Neurobasal, 1x B-27 supplement, 1x NEAA, 2 mM Glutamine, 0.1 mg/ml Pen/Strep [all Gibco, USA], 1x MycoZap-Plus-CL [Lonza, Switzerland], 10 ng/ml human NT-3 [PeproTech, USA], 10 ng/ml human BDNF [R&D Systems, USA]) supplemented with 2 µg/ml doxycycline (Sigma-Aldrich, USA). On day 3, medium was changed to 100% NGN2 medium supplemented with 0.8 µg/ml puromycin (Sigma-Aldrich, USA). On day 5, the selection process was stopped by replacing medium without antibiotics. Medium was changed every other day until day 14, where the cells were harvested for subsequent experiments.

Total RNA sequencing

Bulk RNA-sequencing was carried out for the following samples (each done in biological triplicates). Dataset 1: WT/WT iPSCs, NPCs, d28 cerebral organoids, and d49 cerebral organoids, 70Q/70Q iPSCs, NPCs, d28 cerebral organoids, and d49 cerebral organoids, WT/70Q NPCs. Dataset 2: NGN2 neurons from C1, C2, C3, HD1, HD2, and HD3. Total RNA was isolated using the Qiagen isolation kit and quality-checked by Nanodrop analysis (Nanodrop Technologies). Briefly, total RNA was mixed with 1 µg of a DNA oligonucleotide pool comprising 50-nt long oligonucleotide mix covering the reverse complement of the entire length of each rRNA (28S rRNA, 18S rRNA, 16S rRNA, 5.8S rRNA, 5S rRNA, 12S rRNA), incubated with 1U of RNase H (Hybridase Thermostable RNase H, Epicentre), purified using RNA Cleanup XP beads (Agencourt), DNase treated using TURBO DNase rigorous treatment protocol (Thermo Fisher Scientific, USA) and purified again with RNA Cleanup XP beads. rRNA-depleted RNA samples were further fragmented and processed into strand-specific cDNA libraries using TruSeq Stranded Total LT Sample Prep Kit (Illumina, USA) and sequenced on NextSeq 500, High Output Kit, 1×150 cycles. Raw sequencing reads were mapped to the human genome (GRCh38 assembly) using STAR (version 2.6.0c) aligner⁹⁸. We used the default settings, with the exception of --outFilterMismatchNoverLmax, which was set to 0.05. Reads were counted using the htseq-count tool, version 0.9.1⁹⁹, with gene annotation from GENCODE release 27¹⁰⁰. Differential gene expression analysis was performed using the DESeq2 (version 1.20.00) R package¹⁰¹. All genes with the adjusted P-value lower than 0.05 were considered differentially expressed. Functional enrichment analysis was done using the gProfileR R package¹⁰² (v. 0.6.6) with default settings. All expressed genes were used as background. For heatmap generation for pathway genes of Dataset 1, bulk-RNAseq count matrices were loaded into R (v. 4.2.2). Additional organoid data from neuruloids²⁵ was processed from scRNAseq data to pseudobulk data by randomly pooling single cells into 3 bulk replicates per cell line per condition (HD and WT/WT) and then also loaded into R. The raw matrices were then normalized to counts per millions (CPM) and filtered to only contain genes with more than 1 CPM using edgeR (v. 3.40.2). For the genes corresponding to a pathway of interest, all the different samples and their corresponding control samples were compared with edgeR, yielding the calculated log2fc and adjusted p values with glmLRT based on two-sided likelihood ratio test without multiple comparison adjustments. For each pathway, the obtained results were plotted in a separate heatmap with colors indicating the magnitude of the log2fc and stars indicating the statistical significance. The case of $0.05 > p_{\text{val}} > 0.01$ corresponds to one star, $0.01 > p_{\text{val}} > 0.001$ corresponds to two stars, and everything below corresponds to three stars.

Long-read RNA sequencing

Long-read RNA sequencing was performed using samples from Dataset 1: WT/WT NPCs vs. 70Q/70Q NPCs. We employed full-length poly(A) and mRNA sequencing (FLAM-seq) for high-quality sequencing of entire mRNAs as described before (Legnini et al.). Briefly, total RNA was processed for poly(A) selection with the Truseq mRNA

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preparation kit (Illumina, USA) starting from 10 µg of RNA. For full-length mRNA library preparation, Poly(A)-selected RNA was tailed using the USB poly(A) length assay kit (Thermo Fisher Scientific, USA) in a 20 µl reaction with 14 µl RNA eluted from the poly(A) selection, 4 µl tail buffer mix, 2 µl tail enzyme mix, for 1 h at 37 °C. Reaction was stopped with 1.5 µl of tail stop solution and 1 µl was checked on a picochip to exclude RNA degradation. Tailed RNA was then cleaned up with 1.8× RNAClean XP Beads (Beckmann Coulter, Germany) and eluted in 18 µl water. After reverse transcription, the resulting cDNA was purified with 0.6× XP DNA beads (Beckmann Coulter, Germany) and resuspended in 42 µl of H₂O. The full-length mRNA library was then amplified by PCR with the Advantage 2 DNA polymerase mix (Takara Bio, Japan) according to the following mix: 10 µl 10× Advantage 2SA PCR buffer, 40 µl cDNA, 2 µl 10 mM dNTPs mix, 2 µl 5′ PCR primer 10 µM, IIA, 2 µl 50× Advantage 2 Polymerase mix, 42 µl H₂O and 2 µl of the 10 µM reverse transcription primer-matched universal reverse primer. The PCR reaction was then performed in a thermocycler as follows: 98 °C for 1 min, 23 × (98 °C for 10 s, 63 °C for 15 s, 68 °C for 3 min), 68 °C for 10 min. The amplified library was then purified with 0.6× Ampure XP DNA Beads and resuspended in 42 µl H₂O. One microliter of reaction was checked on a fragment analyzer using High Sensitivity NGS Fragment Analysis Kit (Advanced Analytical Technologies, Germany). For PacBio sequencing the purified PCR libraries were submitted to the Genomics Core Facility of the MDC. Sequencing libraries were prepared using the PacBio Amplicon Template Preparation and Sequencing Protocol (PN 100-081-600) and the SMRTbell Template Prep Kit 1.0-SPv3 according to the manufacturer's guidelines. Sequencing on the SEQUEL IIe SYSTEM (PacBio, Pacific Biosciences of California, USA) was performed in Diffusion mode using the Sequel Binding and Internal Ctrl Kit 2.0. Every library was sequenced on two or three SMRT Cells 1M v.2 with a 1×600 min movie. Circular Consensus Sequence (CCS) reads were generated within the SMRT Link browser 5.0 (minimum full pass of three and minimum predicted accuracy of 90). All primer sequences are reported in Supplementary Data 11.

The bioinformatic analysis of Single Molecule Real Time (SMRT) used a modified IsoSeq3-pipeline followed by an own pipeline of open-source tools and scripts, as described before¹⁰³. For primary analysis, the IsoSeq3-Pipeline from Pacific Biosciences has been used; followed by a secondary Analysis Pipeline with open-source tools and modified scripts. This included mapping to genome with Minimap2¹⁰⁴, collapsing transcripts with cDNA Cupcake and Annotation with SQANTI2¹⁰⁵. Semi-quantitative Analysis of the Long-Read-Transcriptomic Data together with graphical illustrations of the underlying alternative splicing (“exon-intron-skippings”) was performed using a modified TALON-SWAN pipeline. The scripts for the analysis of SMRT-Data are available upon request and were executed on the High-Performance Cluster using BioConda-Environment. As coding editor, “Sublime Text” for Windows (Version Build 4113) was used. The Scripts here contain the essential information on settings, please be aware that not every step (e.g., copying or moving of files) is mentioned. Transcriptomic Long-Read-Data was preprocessed for further usage through a modified pipeline using the TALON tool to enable technology-agnostic long-read analysis pipeline for transcriptome discovery and quantification. Then, using the python library “SWAN”, a library for the analysis and visualization of long-read transcriptomes¹⁰⁶, a reference transcriptome was added and abundance information from the initial SMRT analysis was used to create graphs showing the differential expression between novel and known isoforms within the dataset. Therefore, we defined groups according to the experimental setup and used the transcript information to process the corresponding transcript path. This analysis pipeline is available upon request. Running environment of all Scripts was CentOS 7.7.1908-based at the High-Performance Cluster (HILBERT, Centre for Information and Media Technology (CIM/ZIM) at Heinrich Heine University, Düsseldorf).

Single-cell RNA sequencing

Regionalized midbrain organoids at day 35 were prepared for single-cell RNA sequencing (scRNAseq) from the following samples of Dataset 1: WT/WT and 70Q/70Q. The data were analyzed in three biological replicates, where each replicate was composed of 48 individual mid-brain organoids. Sequencing was carried out in two separate batches. In the first batch, we processed replicate 1 and 2 for WT/WT with replicates 1 and 2 for 70Q/70Q. In the second batch, we processed replicate 3 WT/WT with replicate 3 for 70Q/70Q. In this manner, we could assess the reproducibility of the sequencing results across different independent runs. Midbrain organoids were dissociated using the Papain Dissociation System (Worthington Biochemical, USA). 5 ml of EBSS was added to one papain vial, which was subsequently incubated in a water bath at 37 °C for 10 min. 500 µl of EBSS was then added to one DNase vial and gently mixed. 250 µl of that DNase mix was added to the papain vial resulting in a final concentration of ~20 units/ml papain and 0.005% DNase. About 50–65 organoids were transferred into one well of a 24-well-plate and washed two times with PBS. 2 ml of the pre-warmed papain/DNase solution was added per well, and the plate was placed onto an orbital shaker with the speed set to 65 rpm for 5–10 min at 37 °C. Using a 1 ml pipette, the organoids were gently triturated by mixing up and down and placed back into the incubator for another 10 min for further enzymatic digestion. The digested organoids were then collected in a 15 ml tube and 5 ml of the organoid culture medium was added. By pipetting up and down with a 225 mm polished glass pipette (Brand GMBH, Germany), the mixture was triturated until mostly dissociated. Any piece of not dissociated tissue was allowed to settle to the bottom of the tube. The cell suspension was then transferred to a new 15 ml tube and filtered through a 30 µm cell strainer (Miltenyi biotech, Germany). The cell concentration was determined, and the cells were pelleted by centrifuging at 300×g for 5 min at room temperature (RT). After aspirating the supernatant, the pellet was re-suspended in 200 µl ice-cold PBS. 800 µl of freezing-cold 100% methanol was added to the cell suspension and stored at –80 °C until further analysis. Samples after rehydration were subjected to scRNAseq using the 10× Genomics Chromium Single Cell 3′ Gene Expression system with feature barcoding technology for cell multiplexing. Libraries were generated using 10x Chromium and sequenced with Illumina NovaSeq 6000 platform (Illumina, USA). We processed demultiplex, raw paired-end scRNAseq data using Cell Ranger (v. 7.1.0) from 10x Genomics (<https://www.10xgenomics.com/support/software/cell-ranger/latest>). We used the “cell ranger count” function to align reads to the human genome (GRCh38) and generate digital gene expression (DGE) matrices for each sample. DGEs were further processed in R (v. 4.2.2) using Seurat (v. 5.0.2). Raw DGEs were imported using the function “Read10x” and only genes detected in at least 5 cells were kept for downstream analyses. Similarly, cells with less than 500 genes and 1500 unique transcripts were discarded. After merging all samples in a single Seurat object, we performed gene expression normalization, scaling and computed principle components for each sample independently, followed by data integration using the function “IntegrateLayers” and the “CCAIntegration” method. Then, we performed unbiased clustering and removed clusters 11 and 17 due to abnormally elevated ribosomal and mitochondrial gene expression, respectively. Finally, we performed dimensionality reduction using the “RunUMAP” function and repeated the unbiased clustering step. Clusters were manually annotated in 4 main cell types analyzing their marker genes identified using the “FindAllMarkers” function. Plots were generated with ggplot2 (v. 3.5.0) and piping using dplyr (v. 1.1.4).

Proteomic analysis

We carried out label-free quantification (LFQ) proteomics with sample preparation according to a published protocol with minor modifications¹⁰⁷. We used biological triplicates of Dataset 1: i) WT/WT iPSCs, ii) WT/WT NPCs, iii) 70Q/70Q iPSCs, iv) 70Q/70Q NPCs. We also

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used biological triplicates of Dataset 2: i) NGN2 neurons C1, ii) NGN2 neurons C2, iii) NGN2 neurons C3, iv) NGN2 neurons HD1, v) NGN2 neurons HD2, vi) NGN2 neurons HD3. We lysed all samples under denaturing conditions in buffer with 3 M guanidinium chloride (GdmCl), 5 mM tris-2-carboxyethyl-phosphine, 20 mM chloroacetamide, and 50 mM Tris-HCl at pH 8.5. We denatured lysates at 95 °C for 10 min in a thermal shaker at 1000 rpm, and sonicated them in a water bath for 10 min. A small aliquot of cell lysate was used for the BCA assay to quantify protein concentration. We diluted the lysates (100 µg proteins) using a dilution buffer containing 10% acetonitrile and 25 mM Tris-HCl, pH 8.0, to reach a 1 M GdmCl concentration. We digested proteins with 1 µg LysC (MS-grade, Roche, Switzerland) while shaking at 700 rpm at 37 °C for 2 h. The digestion mixture was diluted with the same dilution buffer to reach 0.5 M GdmCl. We added 1 µg trypsin (MS-grade, Roche, Switzerland) and incubated the digestion mixture in a thermal shaker at 700 rpm at 37 °C overnight. We used solid phase extraction (SPE) disc cartridges (C18-SD, Waters, USA) for peptide desalting, according to the manufacturer's instructions. We reconstituted desalted peptides in 0.1% formic acid in water and further separated them into four fractions by strong cation exchange chromatography (SCX, 3 M Purification, Meriden, USA). We next dried the eluates in a SpeedVac, dissolved them in 20 µl 5% acetonitrile and 2% formic acid in water, briefly vortexed them, and sonicated them in a water bath for 30 s prior to injection into nano-LC-MS. We carried out LC-MS/MS by nanoflow reverse phase liquid chromatography (Dionex Ultimate 3,000, Thermo Fisher Scientific, USA) coupled online to a Q-Exactive HF Orbitrap mass spectrometer (Thermo Fisher Scientific, USA). LC separation was performed with a PicoFrit analytical column (75 µm ID × 55 cm long, 15 µm Tip ID; New Objectives, Woburn, MA, USA) in-house packed with 3 µm C18 resin (Reprosil-AQ Pur, Dr. Maisch, Germany). We eluted peptides using a gradient from 3.8 to 40% solvent B in solvent A over 120 min at 266 nl per minute flow rate. Solvent A was: 0.1% formic acid, while solvent B was: 79.9% acetonitrile, 20% H₂O, 0.1% formic acid. Nanoelectrospray was generated by applying 3.5 kV. A cycle of one full Fourier transformation scan mass spectrum (300–1750 m/z, resolution of 60,000 at m/z 200, AGC target 1e6) was followed by 12 data-dependent MS/MS scans (resolution of 30,000, AGC target 5e5) with a normalized collision energy of 25 eV. We used a dynamic exclusion window of 30 s to avoid repeated sequencing of the same peptides. We sequenced only peptide charge states between two and eight. Raw MS data were processed with MaxQuant software (v1.6.10.43) and searched against the human proteome database UniProtKB with 20,600 entries, released in 05/2020. Parameters of MaxQuant database searching were a false discovery rate (FDR) of 0.01 for proteins and peptides, a minimum peptide length of 7 amino acids, a mass tolerance of 4.5 ppm for precursor, and 20 ppm for fragment ions. We used the function “match between runs”. A maximum of two missed cleavages was allowed for the tryptic digest. We set cysteine carbamidomethylation as fixed modification, while N-terminal acetylation and methionine oxidation were set as variable modifications. We excluded any contaminants from further analysis, as well as any proteins identified by site modification or derived from the reversed part of the decoy database. We report the MaxQuant processed output files, peptide and protein identification, accession numbers, percentage sequence coverage of the protein, *q*-values, and LFQ intensities in Supplementary Data 4, Data 5, Data 8. We performed the correlation analysis of biological replicates and the calculation of significantly different metabolites and proteins using Perseus (v1.6.14.0). We transformed by log₂ the LFQ intensities originating from at least two different peptides per protein group. We employed only protein groups with valid values within compared experiments. We carried out statistical analysis by a two-sample two-tailed t-test with Benjamini-Hochberg (BH, FDR of 0.05) correction for multiple testing. For comprehensive proteome data analyses, we applied gene set enrichment analysis (GSEA)¹⁰⁸. This was carried out to

determine if a priori defined sets of proteins show statistically significant and concordant differences between mutations and controls. For GSEA analysis, we used all proteins with ratios calculated by Perseus. We applied GSEA standard settings, except that the minimum size exclusion was set to 5 and Reactome (v. 7.2) and KEGG (v. 7.2) were used as gene set databases. The cutoff for significantly regulated pathways was set to be ≤0.05 p-value and ≤0.25 FDR. We report the results of the GSEA analysis in Supplementary Data 9.

Metabolomics analysis

Metabolites were analyzed by a targeted LC-MS approach. Metabolite extraction was performed as reported previously¹⁰⁹. We harvested biological triplicates of Dataset 2: i) NGN2 neurons C1, ii) NGN2 neurons C2, iii) NGN2 neurons C3, iv) NGN2 neurons HD1, v) NGN2 neurons HD2, vi) NGN2 neurons HD3. We aspirated the culture medium, quickly rinsed the cells twice with ice-chilled 1x PBS, pelleted the cells, and shock-froze them in liquid nitrogen. A portion of the protein pellets were used in BCA protein assay for normalization among samples. We extracted the metabolites with methyl tert-butyl-ether (MTBE), methanol, and water. The protein pellets were reconstituted in water, acetonitrile, and 50% methanol in acetonitrile. To each sample we added an internal standard mixture containing chloramphenicol, C13-labeled L-glutamine, L-arginine, L-proline, L-valine, and uracil (10 µM final concentration). We used SpeedVac to dry the aliquots. We dissolved dry residuals in three different solvents: i) 100 µl 50% acetonitrile in MeOH with 0.1% formic acid, ii) 100 µl MeOH with 0.1% formic acid for analysis by HILIC column, or iii) 100 µl water, 0.1% formic acid for C18 column mode. We transferred the supernatants to micro-volume inserts. We injected 20 µl per run for subsequent LC-MS analysis. Each sample was finally analyzed in 15 individual LC-MS runs with specific settings for ion polarity, buffers, flow rates, and columns and their temperatures. Over 400 metabolites were selected to cover most of the important metabolic pathways in mammals, based on previous studies¹¹⁰. Since metabolites are very diverse in their chemical properties, we used two different LC columns for metabolite separation: Reprosil-PUR C18-AQ (1.9 µm, 120 Å, 150 × 2 mm ID; Dr. Maisch, Germany) and zicHILIC (3.5 µm, 100 Å, 150 × 2.1 mm ID; Merck, Germany). We used the settings of the LC-MS instrument, 1290 series UHPLC (Agilent Technologies, USA) online coupled to a QTrap 6500 (Sciex, USA)¹¹⁰. The buffer conditions were A1, 10 mM ammonium acetate, pH 3.5 (adjusted with acetic acid); B1, 99.9% acetonitrile with 0.1% formic acid; A2, 10 mM ammonium acetate, pH 7.5 (adjusted with ammonia solution); B2, 99.9% methanol with 0.1% formic acid. We prepared all buffers in LC-MS grade water and organic solvents. We performed peak integration with MultiQuant™ software v.2.1.1 (Sciex, USA) and reviewed it manually. We normalized peak intensities, first against the internal standards, and subsequently against protein abundances obtained from the BCA assay. We used the first transition of each metabolite for relative quantification between samples and controls. Statistical analysis was carried out with Perseus¹¹¹. We employed a two-sample two-tailed t-test with Benjamini-Hochberg (BH, FDR of 0.05) correction for multiple testing. We provide the list of all metabolites including MRM ion ratios, KEGG and HMDB metabolite identifiers, and statistical values in Supplementary Data 10. These data were obtained using a previously reported LC-MS method containing the list of metabolites, transitions, and retention times¹¹⁰.

Proteomic-driven functional metabolic analysis

For the functional metabolic analysis of the proteomic Dataset 1 (including iPSCs and NPCs from WT/WT and 70Q/70Q), we used the Quantitative System Metabolism (QSM) pipeline developed by Doppelganger Biosystem GmbH. We used the approach as described before⁴⁴. QSM data analysis used quantitative information on the expression levels of metabolic proteins (enzymes) to determine metabolic profiles, metabolic states and capacities, and metabolic

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fluxes. To ensure data quality, we used the quality control (QC) score, which counts the number of proteins of interest found, and the QSM score, which evaluates the number of metabolic processes associated with the enzymes found. For our dataset, the QC score was about 80% and the QSM score was around 100%, indicating excellent data quality that ensures reliable interpretability of the results. The kinetic model includes major cellular metabolic pathways of energy metabolism in neuronal cells, as well as key electrophysiological processes at the inner mitochondrial membrane, the mitochondrial membrane potential membrane, the transport of various ions, and the utilization of the proton motive force. Maximal enzyme activities ($V_{\max}$ values) were estimated based on functional characteristics and metabolite concentrations of healthy neuronal tissues¹². Individual metabolic models were inferred using protein intensity profiles. The maximal activities ($v_{\max}$) mean control for the normal state were previously calculated¹². QSM was used to calculate maximal energetic capacity. Energetic capacity was assessed under saturating glucose and oxygen concentrations, corresponding to healthy physiological conditions. Energetic capacities were evaluated by computing the changes of metabolic state elicited by an increase of the ATP consumption rate above the resting value. Maximal neuronal glucose uptake rate was assessed by increasing plasma glucose concentration in a systematic manner as the only energy delivering substrate, assuming high energy demands, and providing saturating oxygen concentrations.

Integration of transcriptomics, proteomics, and metabolomics from NGN2 neurons

Integration analysis was performed for Dataset 2: NGN2 neurons from control iPSCs (C1, C2, C3) vs. NGN2 neurons from HD patient iPSCs (HD1, HD2, HD3). For enrichment analysis, over-representation analysis (ORA) of KEGG pathways of either transcriptomics or proteomics data was performed with Bioconductor clusterProfiler (v.3.0.4), using the significantly up- or down-regulated molecules (adj. p value ≤ 0.05 and $\log_2\text{FC} > 0$ or $\log_2\text{FC} < 0$, respectively). Dysregulated pathways with a set size of 2 to 500 genes and a p value ≤ 0.05 were considered as significantly dysregulated. Networks of dysregulated pathways were constructed via Bioconductor Enrichplot v1.10.2 over-representation analysis (ORA) for human disease ontologies was performed using the Bioconductor DOSE package, and the top 5 enriched terms with p value ≤ 0.05 were plotted in circular networks along with their associated genes. For Gene Set Enrichment Analysis (GSEA), all transcripts or proteins were loaded without prior filtering in clusterProfiler v.3.0.4 and analyzed for associated biological processes (GO BPs). Terms with an adj. p -value ≤ 0.05 and more than 30 associated genes were considered significantly dysregulated. Term redundancy was removed setting the cut-off at 0.6. The top 20 dysregulated BPs were plotted according to their normalized enrichment score (NES). For co-expression analysis, protein co-expression analysis was performed using the ProtExa tool¹³. Briefly, normalized protein expression data was uploaded and analyzed according to the steps indicated in the platform. No further normalization method was applied, while proteins were filtered for a p -value ≤ 0.05 . Top-scored proteins obtained from pre-processing steps were annotated for the construction of co-expression networks using the ARACNE algorithm¹⁴. Co-expressed proteins were clustered with the Walktrap algorithm and edge weight calculation. The clustered network containing the proteins of interest was imported into Gephi (v. 0.10.1)¹⁵, and analyzed further for community detection with a 2.5 resolution. Enrichment analysis for potential transcription factors of co-expressed proteins was performed through TFEA.ChIP tool kit¹⁶ using the default parameters, while gene-disease associations were retrieved from GeDiPNet database¹⁷ through the EnrichR platform¹⁸. For metabolomics analysis, metabolites detected by LC-MS/MS were analyzed for their chemical similarity and clustered based on their class using the ChemRICH tool¹⁹. Significantly dysregulated metabolites with a p value ≤ 0.05

were annotated in the ConsensusPathDB²⁰ for enrichment analysis of affected biological pathways against KEGG, Reactome, SMPDB, EHMN, INOH and HumanCyc databases^{21–26}. Interactions of Significantly dysregulated metabolites were obtained using the MetaboAnalyst platform (v. 5.0)²⁷. OmicsNet platform (v. 2.0) was used for the integration of transcriptomics, proteomics, and metabolomics data²⁸. Significantly dysregulated transcripts (adj. p -value ≤ 0.05), proteins (adj. p -value ≤ 0.05), and metabolites (p -value ≤ 0.05) were uploaded as input lists. Interactions for single- and multi-modalities were predicted using multiple databases. Specifically, for proteins, protein-protein interactions were retrieved from STRING²⁹ with a 0.9 confidence score and protein-metabolite interactions were retrieved from KEGG. Edges were generated only for the input lists without adding new nodes. For transcripts, miRtarBase³⁰ and ENCODE³¹ provided information for mRNA-miRNA and TF-mRNA interactions, respectively, while Recon3D³² was used for metabolite interactions. The Prize-Collecting Steiner Forest (PCSF) algorithm³³ was applied to minimize the overall cost of including edges. The outcome was a subnetwork of the most informative nodes representing the backbone of the organization and functionality of the initial network. Nodes were annotated in KEGG and Reactome for single-omics or multi-omics enrichment analysis. Network visualization was made using the 3D BioLayout in Cytoscape (v. 3.9.1)^{34,35}.

siRNA-based knock-down experiments and branching analysis

For short interfering RNA (siRNA)-based knock-down experiments, siRNA (Dharmacon, USA) was incubated with JetPRIME transfection reagent (Polyplus-transfection, France) and μ Clear black 384-well-plates (Greiner Bio-One, Austria) were coated with 1 pmol siRNA per well. The plates were dried in a SpeedVac and stored at 4 °C wrapped with parafilm until use. Before adding the neurons on the plates, they were additionally coated for 1 h at 37 °C with ready-to-use Geltrex (Gibco, USA). 13 days-old neurons differentiated from the BIHi-005-A-24 line were detached with Accutase (Sigma-Aldrich, USA) for 3 min and collected. The cells were centrifuged at 100 $\times g$ for 5 min and resuspended in neuronal medium. We quantified neuronal branching using our high-content analysis (HCA) assay⁵³. Briefly, 5,000 neurons were seeded per well to the 384-well plate and cultured for 4 days. At day 17 of differentiation, the neurons were fixed by adding 8% PFA (Thermo Fisher Scientific, USA) to the medium and incubated for 20 min. After washing 3 times with PBS (Gibco, USA), 0.05% sodium azide (Sigma-Aldrich, USA) in PBS solution was added to the wells and the plate was stored at 4 °C wrapped with parafilm until staining. For staining, the cells were blocked for 1 h at RT with blocking solution (PBS [Gibco, USA], 3% BSA, 0.05% sodium azide, 0.5% Triton-X-100 [all Sigma-Aldrich, USA]). Then, they were treated with primary antibodies anti-MAP2 and anti-SMI312 in blocking solution over night at 4 °C wrapped with parafilm. Afterwards, they were washed 3 times with PBS and treated with secondary antibodies against guinea pig (AF 488) and mouse (AF 568) and Hoechst 33342 (1:2500; Life Technologies, USA) in blocking solution for 1 h at RT. After 3 washes with PBS, the wells were filled with 0.05% sodium azide in PBS and stored at 4 °C wrapped with parafilm. They were imaged with the high-content microscope Operetta (PerkinElmer, USA) with a 0.4 NA 20 $\times$ air objective and 25 images per well. The images were analyzed with the open-source software CellProfiler (v. 4.2.1) with respect to the axon and dendritic growth and arborization. The data was summarized by calculating the median per well and to compare different plates, the numbers were compared to the median of the control wells (non-targeting scrambled siRNA). siRNA sequences and antibodies are reported in Supplementary Data 11.

PCR analyses

For HTT PCR, genomic DNA was harvested from iPSCs colonies with Phire Animal Tissue Direct PCR Kit (Qiagen, USA). Exon 1 of the HTT

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locus was amplified by PCR using AmpliTaq Gold 360 DNA polymerase (Thermo Fisher Scientific, USA) and HTT-specific primers using following PCR conditions: 95 °C 10 min, 35 cycles: 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min. Products were visualized by agarose gel electrophoresis, individual DNA bands were excised and purified using The Wizard® SV Gel and PCR Clean-Up System (Promega), cloned with CloneJET PCR Cloning Kit (Thermo Fisher Scientific, USA), and submitted to LGC Genomics for Sanger sequencing. Chromatograms were analyzed using CLC Genomics Workbench (Qiagen, USA). For quantitative real-time PCR (qPCR) analysis of cerebral organoids, RNA extraction was performed from four cerebral organoids per condition with RNeasy kit (Qiagen, USA). cDNA was generated from 100 ng RNA using the first strand cDNA synthesis kit (Thermo Fisher Scientific, USA). qPCR experiments were conducted for three technical replicates and three biological replicates with the SYBR green Mastermix (Thermo Fisher Scientific). Amplification of 10 ng RNA was performed in a Vii7 Real-Time PCR system (Thermo Fisher Scientific) as follows: 2 min activation step at 50 °C, followed by 10 min at 95 °C, 40 cycles of denaturation for 15 s at 95 °C, and annealing for 1 min at 60 °C, finalized with 15 s at 95 °C, 1 min at 60 °C, and 15 s at 95 °C. After averaging the CT-values of the three technical replicates, the expression levels of the genes of interest were normalized relative to the average expression of housekeeping genes GAPDH, OAZ1 and ACTB using the delta-delta-CT method. All primer sequences are reported in Supplementary Data 11.

Immunostaining

Cerebral organoids were fixed with 4% PFA for 20 min at RT and washed 3x with PBS for 15 min. Next, organoids were cryoprotected overnight in a 30% sucrose in PBS solution. Following this, organoids were embedded in a 10% sucrose/13 % gelatin solution in PBS and stored at -80 °C upon further use. Hereafter, sections of 20 µm were cut using a Leica CM3050 S cryostat, collected on Superfrost Plus slides and stored at -80 °C. Next, sections were washed three times with warm PBS for 10 s to dissolve and remove any remaining gelatin. Subsequently, sections were fixed again with 4% PFA for 20 min at RT, washed 3x for 10 min with PBS, and blocked for 1 h at RT in blocking solution (PBS, 10% normal donkey serum, 1% Triton X-100). Sections were incubated overnight at 4 °C with the primary antibodies diluted in blocking solution, washed 4x for 10 min with 0.1% Triton X-100 and 0.05% Tween-20 in PBS, and incubated for 1–2 h at RT with Hoechst 33342 (1:2500; Life Technologies, USA) and the secondary antibodies diluted in blocking solution. Finally, sections were washed 4x for 10 min with 0.1% Triton X-100 and 0.05% Tween-20 in PBS, mounted, air-dried and images were taken using the confocal microscope Zeiss Z1 (Zeiss, Germany), Olympus Fluoview 3000 (Olympus, Japan) or ZEISS Axio Observer with an apotome 3 (Zeiss, Germany). NPCs and midbrain organoids were stained following the same procedure. NPCs grown on Matrigel-coated (Corning, USA) coverslips and free-floating midbrain organoids were fixed with 4% PFA (Science Services, Germany) for 20 min at RT and washed three times with PBS. For permeabilization, cells or organoids were incubated with blocking solution (PBS [Gibco, USA], 10% normal donkey serum, 1% Triton-X-100, 0.05% Tween-20 [all Sigma-Aldrich, USA]) for 1 h at RT. Primary antibodies were diluted in blocking solution and incubated overnight at 4 °C on a shaker. Next, the cover slips or organoids were washed three times with PBS and incubated for 1 h at RT with Hoechst 33342 (1:2500; Life Technologies, USA) and the secondary antibodies in blocking solution. After incubation, cover slips or organoids were washed three times with PBS. Finally, cover slips were mounted, air-dried and images were acquired using the confocal microscope Olympus Fluoview 3000 (Olympus, Japan) or ZEISS Axio Observer with an apotome 3 (Zeiss, Germany). Images of free-floating midbrain organoids were acquired using the ZEISS Axio Observer with an apotome 3 as a z-stack, which were then deconvoluted using the Zeiss blue software (v. 3.1) default settings and z-projected with maximum

intensity. Cortical organoids were fixed with 4% PFA for 1 hr at RT and washed 3x with PBS for 15 min. Cortical organoids were embedded into tissue molds that were filled with 2% low melting point agarose. The agar blocks containing the organoids were sliced using a Leica VT1000 S vibratome in ice-cold PBS at 50 µm thickness. Free floating sections were gently transferred to well plates containing PBS and stored at 4 °C until time of staining. Staining and imaging were done in the same manner as the midbrain organoids. Detailed information about antibodies used can be found in Supplementary Data 11.

CHCHD2 and TOM20 imaging quantification

Images of NPCs and cerebral organoids stained against TOM20, CHCHD2, and Hoechst were processed as follows. For NPCs, we analyzed at least two biological replicates, with 11 images being taken for each replicate. For the cerebral organoids, we analyzed at least two biological replicates, with at least three slices of each organoid and three images per slice. In total, we analyzed 77 individual images for 70Q/70Q and 35 individual images for WT/WT. We performed background subtraction using the rolling ball algorithm implementation of Scikit-image¹³⁶ with a radius of 50 pixels. We applied Gaussian blur with a radius of three pixels, to smoothen the images. To separate signal from background, we used Otsu's thresholding method on the respective color channels. We then calculated the amount of colocalized pixels above the threshold for each marker. For comparing amounts of active TOM20 or CHCHD2 signals of different data sets, the amounts of positive signal per image were normalized by the amount of Hoechst signal per image to account for the fact that different images contain different number of cells. Mann-Whitney U tests were used to test for differences between data sets (cell lines or treatments). For mitochondrial network morphology assay of cerebral organoids, pictures of 100x magnification were taken of TOM20 stained sections using a Zeiss Z1 microscope. Next, the pictures were analyzed using ImageJ, as described before⁴¹. Briefly, background was reduced, local contrast enhanced, pictures were made binary and using a "tubeness" plugin mitochondrial structures were tubed. After bandpass filtering, a threshold was set to minimize extremely small and large structures. Lastly, particles were analyzed, yielding information about the size and morphology of the structures. Small mitochondrial structures range were defined as <0.5 µm and large mitochondrial structures as >0.5 µm.

Western blotting of HTT and filter retardation assay (FRA)

Samples from NPCs and guided brain organoids were resuspended in 150 µl RIPA buffer (150 mM NaCl, 50 mM Tris, 0.5% w/v sodium desoxycholate, 1% v/v Triton X-100, 0.1% SDS, before using freshly added 1 mM PMSF and Protease Inhibitor Cocktail 1:100). Cell suspensions were pipetted 5x by using syringes to pass through 27 G needles. The lysates were incubated at 4 °C for 1 h on a turning wheel followed by repeating the step of 27 G needle treatment. The lysates were centrifuged at 15,000 x g for 15 min at 4 °C. The supernatants were transferred to tubes and the protein concentrations were measured by using a BCA Protein Assay kit (Thermo Fisher Scientific, USA). For western blot, 5 µg proteins of each sample were used for detection. In these experiments, polyacrylamide gels consisting of a 4% stacking gel and a 4–12% discontinuous resolving gel were applied. After SDS-PAGE, the proteins were transferred onto nitrocellulose membrane (Amersham Protan 0.45 µm NC, GE Healthcare Life science) by tank blotting either at 500 mA, RT for 1.5 h or 100 mA, 4 °C for 16 h. Proteins on the membranes were detected by using anti-HTT (Anti-Huntingtin antibody [EP5526] (ab109115) Abcam; 1:5,000), primary antibody followed by anti-rabbit-HRP (Abcam; 1:10,000) secondary antibody. For Filter Retardation Assay (FRA), 3 µg protein of each sample was loaded. Both cellulose acetate membrane (Cellulose Acetate Membrane Filter 0.2 Micron, STERILTECH Corporation) and nitrocellulose membrane were equilibrated in PBS/0.01% SDS. The dot blot apparatus was

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assembled with either membrane and the pump system was connected and turned on continuously. After washing the membranes with PBS, the samples were applied followed by two washing steps. The membranes were dried and proteins on the membranes detected with the same antibodies applied in western blot. The quantified figures were analyzed, and all signals were evaluated in ImageJ (v. 1.53a). The signals were calculated by considering white as a maximum value of 65536, and black as a minimum value of 0. All experiments were repeated three times. The values in the figures indicate the average values of all repeats and replicates.

Western blotting of CHCHD2

Cell pellets were re-suspended in 50 μ l of lysis buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% TritonX-100 + 1x cOmplete™, EDTA-free protease inhibitor cocktail (Roche) and incubated on ice for 30 min. The lysate was then centrifuged at 14,000 $\times$ g for 5 min at 4 °C to remove any cell debris. The supernatant was transferred to new vials and the protein concentration was determined via Bradford assay (ROTI®Quant; Roth). For SDS-PAGE, 25 or 50 μ g of protein per lane was loaded on a 12% acrylamide gel. Proteins were blotted onto a nitrocellulose membrane using semi-dry Western blotting (Trans-Blot Turbo Transfer System; BIO-RAD). The membranes were then blocked with 5% (w/v) milk powder in TBS-T on a shaker at RT for 1 h, followed by incubation with the primary antibody diluted in 3% (w/v) milk powder in TBS-T on a shaker at 4 °C overnight. The next day, the membranes were washed three times for 10 min in TBS-T before being incubated with the secondary antibody diluted in 3% (w/v) milk powder for 4 h at RT on a shaker. After incubation with the secondary antibody, the membranes were washed again with TBS-T before Pierce™ ECL Western Blotting Substrate was added to the membranes. The chemiluminescence signal was detected using the ChemoStar (Intas) or Amersham™ Imager 600 (GE). Western blots were then analyzed using ImageJ (v. 1.53a). CHCHD2 polyclonal antibody (19424-1-AP Proteintech 1:500–1:1000) and beta actin monoclonal antibody (ab8224 Abcam 1:1000 and 66009-1-Ig Proteintech 1:6000) were used as primary antibodies. Goat IgG anti-rabbit IgG (H + L)-HRPO (111-035-003 DIANOVA 1:3000 – 1:5000) and goat IgG anti-mouse IgG (H + L)-HRPO (115-035-003 DIANOVA 1:10000) were used as secondary antibodies.

Electron microscopy

Electron microscopy (EM) was performed at the Core Facility for Electron Microscopy (CFEM) at the medical faculty of the Heinrich Heine University Duesseldorf. NPCs were grown in 15 cm petri dishes coated with Matrigel. When the NPCs were confluent, they were fixed using 3% glutaraldehyde (Serva) buffered with 0.1 M sodium cacodylate (Serva) buffer (pH 7.2). Following fixation, the cells were washed with 0.1 M sodium cacodylate buffer (pH 7.2) and subsequently stained with 1% osmium tetroxide (Science services) for 50 min at RT. This was followed by additional staining with 1% uranyl acetate (Merck)/1% phosphotungstic acid (Merck) in 70% ethanol for 1 h. To facilitate the dehydration process, the samples underwent serial ethanol treatment before being embedded in spur resin and polymerize at 70 °C for at least 48 h. Ultra-thin sections were prepared using an ultra-microtome (EM UC7). Imaging was conducted using a JEM-2100 plus (JOEL) equipped with an EM-24830 flash CMOS camera system (JOEL). EM images were analyzed using ImageJ (v. 1.53a)¹³⁷. In each image, mitochondrial structures were identified and classified. Then, it was determined if cristae are visible, if yes how many and along what axis they were angled. If they were perpendicular to the major axis of the mitochondria, they were classified as transverse, and contrary, if they were perpendicular to the minor axis of the mitochondria they were classified as longitudinal. Next, the mitochondrion was traced with the freehand selection tool in ImageJ and measured regarding size and shape.

Structural analysis of the mitochondrial respiratory chain (MRC)

The steady-state levels of MRC subunits were analyzed by SDS-PAGE of whole cell lysates from NPCs (WT/WT, 70Q/70Q and WT/70Q) solubilized with HEPES extraction buffer (20 mM HEPES pH 7.4, 10 mM NaCl, 10% glycerol, 1% Triton X-100, protease inhibitors) for 30 min on ice. Lysates were centrifuged at 16,000 g for 10 min at 4 °C, protein concentration was measured with the BCA protein assay kit (Pearson), and 15 μ g protein in 1:1 Laemmli 2x (Biorad) were loaded onto 12.5% polyacrylamide SDS-page gels. Gels were blotted onto nitrocellulose membranes and protein immunoblotting was performed with antibodies targeting representative MRC subunits (detailed in Supplementary Data 11). The relative abundance of MRC complexes and supercomplexes was determined by Blue Native (BN)-PAGE from NPCs (WT/WT, 70Q/70Q and WT/70Q)⁴⁸. Briefly, mitochondrial fractions were isolated, protein concentrations were determined, and samples were solubilized with digitonin at a detergent-to-protein ratio of 4:1. Pre-cast NativePAGE™ 3-12% Bis-Tris gels (Invitrogen) were loaded with 25 μ g of mitochondrial protein and processed for blue native electrophoresis. Proteins were then transferred to PVDF membranes at 40 V overnight and probed with antibodies (Supplementary Data 11). Uncropped and unprocessed blot scans are provided in Supplementary Fig. 10.

Bioenergetic profiling

Live-cell assessment of cellular bioenergetics in NGN2 neurons was performed using Seahorse XF96 extracellular flux analyzer (Seahorse Bioscience, USA), as we described before⁸³. Briefly, 20,000 neurons were plated into each Matrigel-coated (Corning, USA) well of the XF96 well plates. Cells were maintained in the plates for 2 weeks. On the assay day, neurons were incubated at 37 °C and 5% CO₂ for 60 min to allow media temperature and pH to reach equilibrium before starting the simultaneous measurement of mitochondrial respiration (oxygen consumption rate, OCR) and anaerobic glycolysis (extracellular acidification rate, ECAR) using the sequential introduction of oligomycin, FCCP, and then rotenone plus antimycin A (all products at 1 μ M; Sigma-Aldrich, USA). Normalization to DNA content in each well of the plate was performed using the CyQUANT Kit (Molecular Probes, USA). The supernatants were stored before and after the seahorse assay and used for lactate measurement using a Lactate Fluorometric Assay Kit (Biovision, USA).

CHCHD2 overexpression

NPCs were transduced with adeno-associated viruses (AAV) carrying a construct to overexpress CHCHD2 (pAAV EF1a CHCHD2 GFP WPRE3; Viral Core Facility Charité, Germany). As a control, AAV which only express GFP were used (scAAV9/pTRs-KS-CBh-EGFP-BGH). These particles were kindly provided by Qinglan Ling and Steven Gray (University of Texas Southwestern Medical Center). NPCs were first seeded onto coverslips coated with Matrigel in a 24-well-plate at a density of 4.8×10^4 cells/well. 24 h later, they were treated with 1.75×10^8 viral particles per well. After another 24 h, the media was exchanged. 2 days later, the NPCs were fixed and subsequently stained against TOM20 and CHCHD2 as described in the section „Immunostaining“. The images were semi-automatically analyzed as follows. All images got a gaussian blur with sigma = 0.5 applied, resulting in a three-pixel blurring radius, to smoothen the images before thresholding. Hoechst and TOM20 images were individually thresholded using Otsu thresholding, and EGFP and CHCHD2 images were thresholded using Triangle thresholding. To be sure that the effect of the AAV transduction is analyzed correctly, only cells with stronger GFP signal than the background fluorescence were considered. The thresholded images were loaded into QuPath¹³⁸, and the cells with a stronger marker were manually annotated. The annotated cells showing stronger GFP signal were exported as JSON files before being converted to segmentation masks. The masks were used during quantification to only analyze the

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respective areas instead of the whole image. Therefore, it was not necessary to use automated background noise subtraction. The images were then quantified as described in the method section “CHCHD and TOM20 quantification”. For mean intensity, the amount of signal (pixels with brightness > 0) was observed. For the area, the amount of signal was observed. The two groups (overexpression and control) were compared using Welch’s *t*-test.

Statistical analysis

Data are expressed as mean and standard deviation (mean ± SD) where normality of the distribution could be verified, or as median and quartiles (median [1st; 4th quartiles]) otherwise. Significance was assessed using parametric tests (Welch’s *t*-test, ANOVA) for normally-distributed data and non-parametric tests (Mann-Whitney *U* test, Kruskal-Wallis) when normal distribution could not be verified. Unless otherwise indicated, data were analyzed using GraphPad-Prism (v. 4.0) (GraphPad Software, USA). Schematics were drawn using Inkscape (v. 1.2.1).

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

There are restrictions to the availability of the patient-derived iPSC lines due to the nature of our ethical approval that does not support sharing to third parties and does not allow to perform genomic studies to respect the European privacy protection law. The datasets generated during this study are available, in cases data protection laws did not prevent the original datasets from being published. Source Data are provided as a Source Data file. • The RNA-sequencing data generated in this study have been deposited in NCBI Gene Expression Omnibus (GEO) database. Bulk RNA sequencing can be found under accession code GSE233916: <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE233916>. • Single-cell RNA sequencing can be found under accession code GSE271852: <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE271852>. • Long-reads RNA sequencing data generated in this study have been deposited in NCBI Sequence Read Archive (SRA) Bioproject repository according to MIAME compliant data submissions and can be found under the accession code PRJNA1138763: <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1138763/>. • The mass spectrometry proteomics data generated in this study have been deposited in the ProteomeXchange Consortium via the PRIDE partner repository¹³⁹ under the accession code PXD041846: <http://www.ebi.ac.uk/pride/archive/projects/PXD041846>. • The mass spectrometry metabolomics data generated in this study have been deposited in Peptide Atlas repository under the accession code PASS04827: <http://www.peptideatlas.org/PASS/PASS04827>. Source data are provided with this paper.

Code availability

Codes used for sRNAseq data analysis and related figure plotting are available on GitHub: https://github.com/rajewsky-lab/Huntington_midbrain_organoids¹⁴⁰. Codes used for CHCHD2 and TOM20 quantification analyses are available on GitHub: https://github.com/Scaramir/HD_colocalization¹⁴¹. The pipeline for the analysis of neurite outgrowth with the open-source software CellProfiler is available here: <https://zenodo.org/records/6642365>⁵³.

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3.4. BALANCING NEURONAL ACTIVITY TO FIGHT NEURODEVELOPMENTAL DISEASES

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Abstract

In a recent study, [Rylaarsdam and colleagues](#) revealed that mutant PACS1 gene, which causes a rare neurodevelopmental syndrome, affects the firing ability of human neurons without dysregulating the cellular architecture of brain organoids. These findings suggest aberrant neuronal electrophysiology as a possible interventional target for pediatric diseases impairing brain development.

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Spotlight

Balancing neuronal activity to fight neurodevelopmental disorders

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Alessandro Prigione ^{1,*}

In a recent study, Rylaarsdam and colleagues revealed that mutant PACS1 gene, which causes a rare neurodevelopmental syndrome, affects the firing ability of human neurons without dysregulating the cellular architecture of brain organoids. These findings suggest aberrant neuronal electrophysiology as a possible interventional target for pediatric diseases impairing brain development.

Neurodevelopmental diseases encompass a wide range of conditions that affect the development and function of the CNS, leading to cognitive, behavioral, and neurological impairments. A struggle in studying neurodevelopmental disorders lies in their heterogeneity, since they can arise from a variety of genetic, environmental, and epigenetic factors, making it challenging to identify common underlying mechanisms and develop targeted therapies. Understanding how specific genetic factors could contribute to impaired brain development is therefore a crucial step towards establishing effective interventions [1].

PACS1 syndrome is a rare neurodevelopmental syndrome characterized by distinguishable craniofacial abnormalities, developmental delays, and intellectual disability. The disease was first identified in 2012 when two patients presenting with similar symptoms shared the same single *de novo* c.607C>T variant in the

phosphofurin acidic cluster sorting protein 1 (PACS1) encoding gene, which results in an arginine to tryptophan (R203W) substitution [2]. Of the nearly 200 individuals who have been so far diagnosed, all but one patient possess the same p.R203W variant, which makes this quite a unique disorder.

The PACS1 protein is found in a complex system of intracellular membranes known as the trans-Golgi network, which sorts proteins and other molecules and sends them to their intended destinations. PACS1 protein is broadly expressed in several tissues, including the CNS, and has been linked to a variety of cellular processes such as vesicle trafficking, ion transport, DNA replication, RNA binding, and regulations of chromatin stability. However, the specific impact of mutant PACS1 on brain development has remained elusive.

Recently, Rylaarsdam and colleagues investigated the role of PACS1 p.R203W variant on human neurodevelopment by applying an approach based on patient-derived induced pluripotent stem cells (iPSCs) [3]. Using CRISPR/Cas9, the authors generated isogenic iPSC lines by correcting the PACS1 p.R203W variant in patient iPSCs and by introducing the same variant in control iPSCs. Dorsal brain organoids generated from the isogenic iPSC lines did not show overt defects caused by the PACS1 p.R203W variant. Whether young (40 days old) or mature (80 days old), PACS1 p.R203W brain organoids did not display alterations in proliferation, cytoarchitectural organization, or cell-specification dynamics. Single-cell transcriptomics of brain organoids at both time points corroborated the lack of changes in the proportion of cell types. At the same time, the transcriptomic analysis uncovered the dysregulation of ion transport and synaptic signaling genes in mature glutamatergic neurons, together with up-regulation of GABAergic receptor signaling. Accordingly, mature brain organoids

carrying the PACS1 p.R203W variant presented higher GABAergic synaptic density. However, calcium responses of mutant brain organoids did not present significant alterations at the basal level or upon treatment with GABAergic antagonist. These negative results prompted the authors to utilize a complementary two-dimensional approach based on pure glutamatergic neurons generated from iPSCs through forced expression of the transcription factor Neurogenin 2 (NGN2). Electrical activity of PACS1-mutant NGN2 neurons recorded using multi-electrode array (MEA) revealed crucial abnormalities, including prolonged network burst with increased inter-spike interval within bursts and reduced network synchrony over time.

The work by Rylaarsdam and colleagues is a notable step in decoding the impact of PACS1 p.R203W variant on human brain development. The observation of aberrant neuronal firing in the absence of structural organization suggests the possibility that an imbalance in neuronal activity is a cause of neurodevelopmental impairment and possibly a root symptomatology that is shared by other disorders of brain development. Dysregulation of excitatory/inhibitory CNS activity is among the major hypotheses for the etiology of neurodevelopmental disorders, including some forms of autism spectrum disorders (ASD) [4]. Other recent iPSC-based studies in brain organoids and pure GABAergic and glutamatergic cultures carrying ASD-associated gene variants have also identified the presence of excitatory/inhibitory imbalance [5,6]. The culprit for this firing dysregulation is mostly attributed to aberrant GABAergic activity. Accordingly, the GABAergic agonist baclofen has been suggested as potential treatment for severe forms of ASD [7]. The work by Rylaarsdam and colleagues demonstrates that in addition to GABAergic dysfunction, firing defects may also impact glutamatergic neurons (Figure 1). These findings open the possibility that neural activity imbalance may

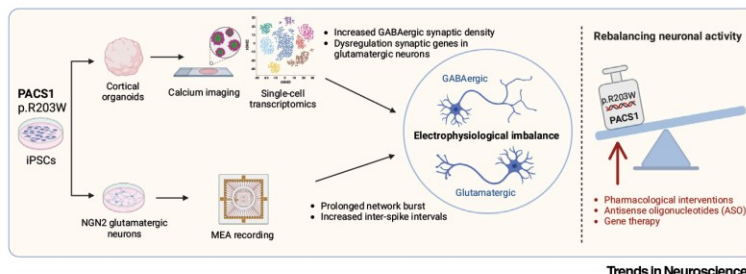


Figure 1. Electrophysiological imbalance in PACS1 syndrome. In a recent study by Rylaarsdam *et al.* [3], the authors used human brain organoids and neuronal cultures from induced pluripotent stem cells (iPSCs) to investigate the neurodevelopmental impact of p.R203W variants in the phosphofurin acidic cluster sorting protein 1 (PACS1) gene, which are associated with the rare neurodevelopmental syndrome PACS1 syndrome. Single-cell transcriptomics and calcium imaging of brain organoids coupled with multi-electrode array (MEA) network analyses in glutamatergic neurons led to identification of aberrant electrophysiology activity and excitatory/inhibitory imbalance caused by the PACS1 p.R203W variant. These findings open the way to therapeutic avenues aiming at counteracting this imbalanced neuronal activity (right). Possible strategies might include pharmacological interventions, gene therapy approaches, or the use of antisense oligonucleotides (ASO) against PACS1. Figure created with [BioRender.com](https://www.biorender.com). Abbreviation: NGN2, Neurogenin 2.

represent a targetable system for neurodevelopmental syndromes that can be tackled using platforms based on genome engineered iPSCs. By leveraging on MEA technology applied to neurons derived from iPSCs carrying various gene mutations associated with neurodevelopmental diseases [6], it may be possible to unveil shared and specific aberrations of neuronal activity and screen for drug modulators.

The findings by Rylaarsdam and colleagues also raise a number of questions. With respect to PACS1 syndrome, one aspect to be clarified is the gene/protein relationship and its contribution to the disorder. The authors observed that PACS1 protein concentration remained constant among control and mutant brain organoids and NGN2 neurons. Hence, disease symptoms may not stem from changes in protein abundance but rather from protein function alterations that could develop through a gain-of-function (GOF) mechanism. In agreement with these observations, a recently generated mouse model of PACS1 syndrome showed no overt alteration of brain structures and similar PACS1 expression [8]. PACS1 mice exhibited Golgi defects that resulted in abnormally increased

neurite arborization, which was recapitulated in mutant iPSC-derived neural progenitors and was ameliorated using antisense oligonucleotides (ASO) targeting PACS1 or HDAC6 [8]. These latter data support the GOF mechanism for PACS1 syndrome. However, NGN2 neurons carrying the PACS1 p.R203W variant investigated by Rylaarsdam *et al.* did not show aberrant arborization. Additional studies using various neuronal types at different time points are warranted to address the possible GOF impact of PACS1 mutations on dendrite arborization and overall neuronal morphogenesis. Lastly, it remains unclear why specific neuronal populations may be more susceptible to PACS1 defects. We speculate that mitochondrial diversity may play a contributory role, given the impact of mitochondrial defects in neuronal activity and brain organoid development and the different mitochondrial features existing within distinct CNS cell types [9,10].

From the work of Rylaarsdam and colleagues, insights are now available into how PACS1 variants could impact human neurodevelopment. Hopefully, these findings will eventually lead to the identification

of effective treatments not only for this single rare syndrome but potentially also for other neurodevelopmental disorders sharing common targetable features.

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Declaration of interests

The authors declare no competing or financial interests related to this work.

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3.5. DEEP LEARNING-DRIVEN NEUROMORPHOGENESIS SCREENINGS IDENTIFY REPURPOSABLE DRUGS FOR MITOCHONDRIAL DISEASE

Menacho C*, Okawa S*, Álvarez-Merz I, Wittich A, Muñoz-Oreja M, Lisowski P, Pentimalli TM, Rybak-Wolf A, Inak G, Zakin S, Thevandavakkam M, Petersilie L, Zaliani A, Modly B, Seibt A, Donnelly J, Woleben K, Fernandez-Checa J, Herebian D, Mayatepek E, Rajewsky N, Spinazzola A, Schuelke M, Perstein E, Rossi A, Distelmaier F, Holt IJ, Pless O, Rose CR, del Sol A, Prigione A

Abstract

Mitochondrial disease encompasses untreatable conditions affecting tissues with high energy demands. A severe manifestation of mitochondrial disease is Leigh syndrome (Leigh), which causes defects in basal ganglia and midbrain regions, psychomotor regression, lactic acidosis, and early death. We previously generated isogenic pairs of Leigh cerebral organoids and uncovered defects in neuromorphogenesis. Here, we leveraged on this disease feature to devise drug discovery pipelines. We developed a deep learning algorithm tailored for cell type-specific drug repurposing to identify drugs capable of promoting neuronal commitment. In parallel, we performed a survival drug screen in yeast and validated the repurposable hits on branching capacity in Leigh neurons. The two approaches independently highlighted azole compounds, Talarozole and Sertaconazole, both of which lowered lactate release and improved neurogenesis and neurite organization in Leigh midbrain organoids. Hence, targeting neuromorphogenesis has led to identify potential new drugs for mitochondrial disease and could prove an effective strategy for further drug discovery.

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Contribution:

Establishment, execution, and analysis of high-content imaging assay to measure neuromorphogenesis defects on patient-derived iNs carrying *SURF1* mutations. Execution and analysis of validation of drug compounds on neuronal morphogenesis.

Establishment, execution, and analysis of drug discovery platform using midbrain organoids carrying *SURF1* mutations (semiautomated image analysis pipeline for organoid size, lactate release, immunohistochemistry analysis and preparation for single-cell RNA sequencing).

Conceptualization and preparation of the manuscript, including figure design, visualization, writing and review.

Deep learning-driven neuromorphogenesis screenings identify repurposable drugs for mitochondrial disease

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Keywords

Mitochondrial disease; Leigh syndrome; brain organoids; neurons; drug screening; morphogenesis

Summary

Mitochondrial disease encompasses untreatable conditions affecting tissues with high energy demands. A severe manifestation of mitochondrial disease is Leigh syndrome (Leigh), which causes defects in basal ganglia and midbrain regions, psychomotor regression, lactic acidosis, and early death. We previously generated isogenic pairs of Leigh cerebral organoids and uncovered defects in neuromorphogenesis. Here, we leveraged on this disease feature to devise drug discovery pipelines. We developed a deep learning algorithm tailored for cell type-specific drug repurposing to identify drugs capable of promoting neuronal commitment. In parallel, we performed a survival drug screen in yeast and validated the repurposable hits on branching capacity in Leigh neurons. The two approaches independently highlighted azole compounds, Talarozole and Sertaconazole, both of which lowered lactate release and improved neurogenesis and neurite organization in Leigh midbrain organoids. Hence, targeting neuromorphogenesis has led to identify potential new drugs for mitochondrial disease and could prove an effective strategy for further drug discovery.

Introduction

Mitochondrial disease includes rare conditions affecting organs with high energy needs such as the brain, which requires highly functional mitochondrial oxidative phosphorylation (OXPHOS).¹ One of the most severe forms of mitochondrial disease in children is Leigh syndrome (Leigh) (OMIM #256000).² Leigh, also known as “infantile subacute necrotizing encephalomyelopathy”,³ causes lesions in the basal ganglia and midbrain, leading to motor impairment, intellectual disability, and lactic acidosis,⁴ with early death following episodes of metabolic decompensation.^{5,6} The most frequently mutated gene in Leigh is *SURF1* (surfeit locus protein 1, NM_003172.2), an assembly factor for complex IV of the mitochondrial respiratory chain.^{7,8} There is a paucity of effective animal models of Leigh⁹, especially of *SURF1* defects, as *Surf1* knock-out (KO) mice failed to develop Leigh-like phenotypes and instead exhibited prolonged lifespan.^{10,11} For this reason, it is currently challenging to identify potential treatments for Leigh.^{12,13}

The prevalent view of the pathomechanisms of Leigh is that the disease develops as early-onset neurodegeneration, where neurons die due to excessive production of free radicals because of ineffective OXPHOS.¹⁴ However, antioxidant therapies failed to show clinical improvements in patients,^{12,13} possibly because antioxidants blunt physiological compensatory responses.¹⁵ Accordingly, we found no redox imbalance in isogenic induced pluripotent stem cells (iPSCs) model of Leigh carrying *SURF1* defects.¹⁶ Instead, we demonstrated an impairment in neuronal morphogenesis.¹⁶ Hence, Leigh mutations may interfere with the ability of neurons to form appropriate connections, leading to faulty wiring and reduced resilience to metabolic stress.¹⁷ Here, we sought to target neuromorphogenesis to devise screening strategies to identify potential candidate compounds for Leigh.

Using single-cell RNA-seq (scRNAseq) datasets of Leigh cerebral organoids (CO), we applied a deep learning (DL) algorithm for cell type-specific drug repurposing and predicted drugs capable of promoting neural commitment in Leigh neural cells. Concurrently, we screened 2,250 FDA-approved drugs for survival in *SURF1*-KO yeast. The two approaches identified the drugs Sertaconazole and Talarozole, both of which improved neuromorphogenesis in Leigh induced neurons (iNs) and in Leigh midbrain organoids (MO), and ameliorated their growth rate, lactate release, and neuronal metabolism. Collectively, our data indicate that neuromorphogenesis is an effective therapeutic target for Leigh.

Results

Neuromorphogenesis screenings in 2D and 3D models of Leigh

We previously demonstrated that Leigh mutations impact the ability of neural progenitor cells (NPCs) to switch to OXPHOS and commit to neuronal fate.¹⁶ Here, we developed 2D and 3D

pipelines to address NPC commitment defects. NPCs were either converted into iNs by overexpressing the transcription factor Neurogenin 2 (NGN2)¹⁸, or instructed to generate MO¹⁹ (**Figure 1A**). Using our high-content analysis (HCA) pipeline to quantify neuromorphogenesis,²⁰ we identified significant defects occurring five days after NGN2 induction in Leigh iNs compared to isogenic control (CTL) iNs (**Figure 1B**; **Figure S1A-B**). We used scRNAseq to benchmark our MO with respect to previously published MO derived from NPCs²¹ (**Figure S1C**). We found that our MO contained a high number of tyrosine hydroxylase (TH)-positive midbrain dopaminergic neurons (mDANs) (31 %) already at day 35 (**Figure 1C-D**; **Figure S1D**), making this early time point suitable for our analyses. When compared to CTL MO, Leigh MO exhibited aberrant size growth (**Figure 1E**), increased release of lactate in the medium (**Figure 1F**), and altered spontaneous calcium activity and metabolic stress-induced calcium signals (**Figure 1G**; **Figure S1E-L**). Morphological evaluation of axons (SMI312-positive) and dendrites (MAP2-positive) within Leigh MO confirmed aberrant neurite organization (**Figure 1H**) coupled with reduction of TH-positive neurons (**Figure 1I-J**).

We sought to establish whether aberrant neuromorphogenesis could represent an interventional target in Leigh. We reanalyzed our published scRNAseq datasets of isogenic Leigh CO¹⁶ to determine key affected populations. Uncommitted precursors showing markers of radial glia (RG) identity were present in both Leigh and CTL CO, while more committed intermediate progenitor cells (IPCs) and neurons were mostly absent in Leigh CO (**Figure 2A-B**; **Figure S2A-B**). We therefore reasoned that to counteract this defective neuromorphogenesis, one could forcibly promote neuronal fate in Leigh cells. Pseudotime analysis confirmed that neuronal differentiation was following this directionality RG > IPCs > neurons (**Figure 2C-D**). We identified differentially expressed transcription factors (DETF) between RG and IPCs and between RG and Neurons (**Figure 2E-F**). We used this defined set of DETFs and a cell type-specific drug perturbation transcriptomics database to develop a DL strategy to uncover drugs capable of triggering cellular conversion from RG directly to neurons (DETF1) or from RG to IPCs and then to neurons (DETF2) (**Figure 2E-F**; **Figure S2C**). This strategy highlighted 11 DL-predicted drugs (DLDs) for DETF1 and 17 DLDs for DETF2. Among these 28 DLDs, we selected 9 based on their available safety profile in humans. In parallel, we carried out a rescue screen in Leigh yeast cultures using a library of 2,250 repurposable drugs. The rescue assay was based on survival of wild-type (WT) and SURF1 homologue SHY1²² knock-out (Δ SHY) yeast strains upon nutrient removal (**Figure S2F-H**). Among the top 50 yeast screening drugs (YSDs) (**Figure S2I**, green dots), we selected 4 YSDs belonging to the class of azole-containing compounds, which was the most strongly affected class among rescuer drugs in Leigh yeast (**Figure S2I**, purple dots). We validated DLD1-9 and YSD1-4 in Leigh iNs to assess their impact on neuromorphogenesis

(**Figure 2G-H; Figure 2J**). We identified DLD8 (Talarozole) and YSD4 (Sertaconazole) as being capable of increasing neuronal number and neurite length in Leigh iNs in a dose-dependent manner (**Figure 2H-K; Figure S2E; Figure S2K**). Since the highest concentrations (50 μ M) of Talarozole and Sertaconazole showed toxic effects (**Figure S2D; Figure S2J**), we focused on lower concentrations for downstream analyses.

Identified drugs Sertaconazole and Talarozole rescue Leigh phenotypes

Next, we aimed to determine the effect of the two identified drugs in Leigh MO (**Figure 3A**). 0.1 μ M Sertaconazole or 1 μ M Talarozole represented the highest concentrations that could be administrated chronically to Leigh MO without loss of viability (**Figure S3A-B**). At these concentrations, the drugs rescued the aberrant growth rate of Leigh MO (**Figure 3B**), reduced their lactate release (**Figure 3C**), promoted the development of TH-positive neurons within Leigh MO, with a significant effect seen for Talarozole (**Figure 3D**), and normalized the disrupted neurite organization of dendrites (MAP2-positive) and axons (SMI312-positive) (**Figure 3E**).

We reasoned that impaired neuromorphogenesis of Leigh might be linked to aberrant mitochondrial motility, as proper anchoring of mitochondria is crucial for neurite branching.^{23,24} Leigh mDANs showed abnormally increased mitochondrial motility with fewer mitochondria that were anchored or stationary (**Figure 3F**). The increase in mitochondrial movements could not be recapitulated by chemical disruption of mitochondrial viability, which instead caused a decrease in mitochondrial motility (**Figure S3D**), suggesting the presence of pathological disruption of mitochondrial anchoring. Indeed, the level of mitochondrial anchoring-related genes was lower in Leigh mDANs and Leigh CO compared to CTL (**Figure S3E**). Leigh MO also displayed reduced expression of mitochondrial anchoring proteins SNPH and KIF5A (**Figure 3G-H**). However, treating Leigh MO with Sertaconazole or Talarozole did not rescue the expression of anchoring proteins (**Figure 3G-H**). Hence, promotion of neuromorphogenesis with Sertaconazole or Talarozole might be beneficial for Leigh neural cells despite the persistence of mutation-related defects.

We then focused on neural metabolism and carried out functional metabolic analyses based on scRNAseq of Leigh CO and CTL CO. This kinetic approach comprises the major cellular metabolic pathways of energy metabolism in neural cells and was previously used from proteomics of CO derived from autism spectrum disorders or Huntington's disease.^{25,26} The technology has been improved to allow its use from scRNAseq datasets. Metabolic genes overlapped between Leigh CO and CTL CO (**Figure S3F-G**), indicating that it was possible to directly compare their functional metabolic regulation (**Figure S3G**). Compared to CTL, Leigh CO showed functional defects in energy metabolism (with reduction in oxygen

consumption rate, ATP production, and glucose uptake) and in lipid-related metabolism (with defective uptake of fatty acids and ketone bodies) (**Figure 4A-E; Figure S3H-K**).

To address the impact of Sertaconazole and Talarozole on energy metabolism, we performed targeted metabolomics in CTL NPCs and Leigh NPCs (**Figure 4F**). Leigh NPCs exhibited increased glycolysis-related metabolites (**Figure S4B**), which were not reduced upon treatment with Sertaconazole or Talarozole (**Figure S4D**). The abnormal levels of tricarboxylic acid cycle (TCA) metabolites such as succinyl-CoA and oxaloacetate (**Figure 4G; Figure S4E**) were instead ameliorated in treated Leigh NPCs (**Figure 4H; Figure S4F**). The beneficial effects were more evident for Talarozole, which also restored the AMP/ATP ratio of Leigh NPCs (**Figure 4G-H; Figure S4A and S4C**).

To assess lipid metabolism, we focused on cholesterol, which is essential for neuronal morphogenesis^{27,28} and can be disrupted in mitochondrial disease.²⁹ Leigh NPCs exhibited lower membrane-bound cholesterol than CTL NPCs (**Figure 4J-K**). Treatment with Sertaconazole or Talarozole for 48 hours significantly increased membrane-bound cholesterol in Leigh NPCs (**Figure 4J-K**). The amelioration was more prominent for Sertaconazole, which is known to modulate the synthesis of ergosterol, the yeast orthologue of cholesterol.³⁰ Accordingly, Sertaconazole increased neutral lipid levels in both CTL and Leigh NPCs (**Figure S4G**).

Lastly, we investigated the molecular structure of Sertaconazole and Talarozole and their *in silico* capacity to bind the main protein target of Talarozole: cytochrome P45026 (CYP26A1/B1) belonging to the RA pathway.³¹ Since the crystal structure of CYP26A1/B1 is not available, we performed the analysis on its homology model CYP120.^{32,33} Sertaconazole and Talarozole showed a biaromatic moiety close to the iron center of heme, with Talarozole positioned in closer proximity to the center than Sertaconazole (**Figure 4L-N, Figure S4I-K**). Thus, both azoles could bind CYP26A1/B1, although with different affinities (**Figure 4M**). To corroborate this common target, we inspected the expression of RA transcripts in Leigh models. Leigh mDANs exhibited upregulation of enzymes metabolizing RA (e.g. *CYP26A/B1*) and downregulation of RA receptors (e.g. *RORB*) (**Figure 4O**). Indeed, treating Leigh iNs for 24 hours with either Sertaconazole or Talarozole was sufficient to repress the elevated expression of *CYP26B1* (**Figure 4P**), indicating that both drugs can modulate this target in Leigh neural cells.

Discussion

Despite the recent advances in the use of iPSC-based disease modeling and drug discovery, most of the treatments assessed in iPSC models of mitochondrial disease have been compounds suggested by previous studies.^{34,35} This indicates that it is not trivial to identify

new therapeutic entities for mitochondrial disease, possibly because of a lack of innovative targets. In Leigh, the culprit for the neuronal pathology is considered to be the death of neurons following oxidative stress.⁵ Antioxidant therapies have however failed to demonstrate clinical efficacy.^{13,36,37} Here, we showed that promoting neuronal generation could represent an effective approach for Leigh. Enabling neuronal growth and proper connectivity might help neurons becoming more resilient against metabolic decompensation.³⁸ This could be especially important for dopaminergic neurons, which are strongly affected in Leigh¹⁴ and are under elevated energy constraints because of their large size and elongated branches.³⁹⁻⁴¹

To investigate neuromorphogenesis as a Leigh target, we developed a DL algorithm to identify repurposable drugs promoting neural commitment. Current tools predicting the transcriptional response to chemical perturbations typically rely on datasets based on cancer cell lines.⁴²⁻⁴⁴ However, cancer cells exhibit signal transduction pathways and metabolic features that are different from those of non-cancer cells⁴⁵⁻⁴⁷ and can therefore lead to false predictions for diseases like Leigh which instead affect neuronal cells. We therefore designed a DL-based drug repurposing algorithm that circumvents this problem by using solely datasets from non-cancer cells.⁴⁸ Moreover, our algorithm predicts first genes and then drugs in a cell-type specific manner, thereby allowing to identify also drugs that are outside of training (as was the case for Talarozole). Second, we conducted a survival screen in Leigh yeast. Yeast screens have been used before in the context of neurological and mitochondrial diseases,^{49,50} but their predictive power requires validations in more complex models.⁵¹ By combining DL prediction and yeast screen with our human 2D and 3D pipelines for neuromorphogenesis, we identified potential repurposable drugs for Leigh.

Sertaconazole and Talarozole shared similarities in chemical structure that might enable them to interact similarly to specific targets. Talarozole is a retinoic acid metabolism blocking agent (RAMBA) and inhibits cytochrome P450 CYP26A1/B1 thereby increasing RA levels in the cell.⁵² Through *in silico* prediction, we found that the antifungal agent Sertaconazole³⁰ might also interact with CYP26, suggesting a similar modulation of CYP26A1/B1, which could ultimately influence the RA pathway to promote neuromorphogenesis (**Figure 4Q**). RA signaling is known for regulating differentiation, organogenesis, and neuronal outgrowth and development.⁵³⁻⁵⁸ Sertaconazole and Talarozole showed additional beneficial effects on neuronal metabolism by ameliorating OXPHOS, which in turn supports neurogenesis,^{17,59-61} and by modulating cholesterol, which could lead to metabolic reprogramming in NPCs to enhance neuronal generation.^{27,28} We conclude that the effects of Sertaconazole and Talarozole could converge to effect neuronal fate determination in Leigh cells (**Figure 4Q**). Other drugs belonging to the azole family are under consideration for neurological diseases.⁶² It is thus tempting to speculate that these azole

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compounds could share similar mechanistic actions on neuronal mitochondria. Indeed, rescuing brain energy metabolism might represent an interventional strategy for common neurodegenerative disorders.⁶³

Altogether, we show that neuromorphogenesis represents an effective target process for drug discovery of mitochondrial disease. Furthermore, our DL algorithm might constitute a cost-effective approach to identify drug repurposing candidates in the context of other incurable neurodevelopmental syndromes.

Limitations of the study

This study was conducted using yeast and iPSC-derived neural models. Additional work is warranted to dissect the systemic *in vivo* impact of these drugs. Sertaconazole and Talarozole are so far only employed topically, even if recent work suggests potential effect for Talarozole and other RAMBAs in reducing inflammation.^{64,65} Since the inflammation modulatory effect of Talarozole is believed to involve the peroxisome proliferator-activated receptor gamma (PPAR γ),⁶⁶ it would be important to assess the interplay of Sertaconazole/Talarozole with the PPAR γ coactivator-1 α (PGC-1 α), which we previously found altered in Leigh models.¹⁶ This work was based only on *SURF1* defects. Additional work is necessary to evaluate whether the two drugs might have positive effects also for other Leigh mutations. Lastly, it remains to be seen whether the combination of Sertaconazole or Talarozole together with other proposed treatments for Leigh^{37,67}, including for example the PGC-1 α modulator bezafibrate,^{16,68} may potentially lead to synergistic benefits.

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Author contributions

Conceptualization, A.P., C.M., A.D.S.; Methodology, C.M., S.O., A.W., M.M.O., L.P., M.T., S.Z., D.H., A.Se., G.I.; Formal Analysis, S.O., T.M.P., C.M., A.Z., I.A-M., B.M., A.R-W.; J.D.; Resources, E.M., A.P., A.D.S., P.L., A.Sp., E.P., F.D., A.R., N.R., K.W., J.F-C.; Writing – Original Draft, C.M., A.P.; Writing – Review & Editing, A.P., C.M., S.O., A.D.S., O.P., C.R.R., L.P., A.S., I.J.H., I.A-M.; Supervision, A.P., A.D.S., O.P., I.J.H., C.R.R.; Visualization, C.M., A.P.; Funding Acquisition, A.P., A.D.S., O.P.

Declaration of interest

The authors declare no competing financial or commercial interests. C.M., S.O., A.D.S., and A.P. filed a patent application for the use of Talarozole in mitochondrial diseases. C.M., K.W., E.P, and A.P. filed a patent application for the use of Sertaconazole in mitochondrial diseases.

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Methods

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Alessandro Prigione, MD, PhD (alessandro.prigione@hhu.de)

Materials availability

There are restrictions to the availability of Leigh patient-derived iPSCs used in this study due to the nature of our ethical approval that does not support sharing to third parties without a specific amendment and does not allow to perform genomic studies to respect the European privacy protection law.

Data and code availability

The scRNAseq datasets supporting results in this paper are publicly available. The accession numbers are listed in the **Key Resources Table**.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Induced pluripotent stem cells (iPSCs) and neural progenitor cells (NPCs)

Leigh iPSCs and isogenic control iPSCs were obtained before using Leigh patient-derived cells and CRISPR/Cas9 editing.¹⁶ Leigh iPSCs carried the *SURF1* mutation c.769G>A (p.G257R) on both alleles in either a Leigh patient background or a healthy control background. The healthy iPSC line was obtained from Dr. Heiko Lickert (Helmholtz Center Munich).⁶⁹ Isogenic control iPSCs carried no *SURF1* mutation in either the patient background or the control background. All iPSC lines were used in accordance with the Ethical Approval obtained by the Medical Faculty of the Heinrich Heine University (Study Number 2020-967_5). iPSC-derived Leigh NPCs and isogenic control NPCs used in this study were generated and characterized before.¹⁶ All NPCs were cultured as described there, with minor modifications. NPCs grew in matrigel-coated 6-well plates in sm+ medium, consisting of a base media 1:1 ratio of Neurobasal and DMEM-F12 supplemented with N2 (0.5 x), B27 without vitamin A (0.5 x), 2 mM glutamine, Myco-Zap-plus-CL (1 x), 3 µM CHIR 99021, 0.5 µM Purmorphamine, and 150 µM ascorbic acid. Media was exchanged every other day and cells were passaged weekly at a ratio 1:6 to 1:10 using accutase treatment. All NPCs were used in experiments between passage 10 and 25. All cells were routinely monitored to ensure lack of mycoplasma contamination.

METHOD DETAILS

Generation of midbrain organoids (MO)

MO were generated following a published protocol,¹⁹ with some modifications. To start the generation of MO, NPCs were detached using accutase to obtain a single cell suspension. Cells were then counted using an automatic cell counter, with a gating size of 5-19 μm , and seeded onto low-attachment U-bottom 96-well plates at a density of 9,000 cells/150 μl media per well. Seeding media was composed of a basal media containing 1:1 ratio DMEM-F12:Neurobasal, N2 (0.25 x), B27 without vitamin A (0.25 x), glutamax (1 x), MycoZap-Plus-CL (1 x), 0.4 % (w/v) polyvinyl-alcohol (PVA) supplemented with the following small molecules: 0.5 μM Purmorphamine, 3 μM CHIR 99021 and 100 μM ascorbic acid. After 2 days, seeding media was replaced with a basal media supplemented with 100 μM ascorbic acid, 1 μM Purmorphamine, 1 ng/ml BDNF, and 1 ng/ml GDNF, to induce ventral patterning. Four days later, the media was exchanged to a maturation media, composed of the basal media supplemented with 100 μM ascorbic acid, 2 ng/ml BDNF, 2 ng/ml GDNF, 1 ng/ml TGF- β 3, and 100 μM dibutyl-cAMP. On day 6, a single dose of 5 ng/ml Activin A was added. From day 7 on, maturation media (without Activin A) was refreshed 3 times a week. For experiments with chronic treatment, MO were treated from day 2 until the day of the assay (either until day 9 or day 28-35) with either 0.1 μM Sertaconazole, 1 μM Talarozole or DMSO (vehicle). DMSO samples contained 0.1 % (v/v) DMSO, which is equivalent to the amount of solvent in the samples that received compound treatment. Treatments were refreshed upon every media change every other day.

MO growth rate analysis, viability assay, and lactate release

To measure organoid size, pictures were taken every other day with a Nikon Eclipse TS2 inverted routine microscope using a 4 x objective and ensuring a good contrast between the background and the organoid. Organoid area and perimeter were measured using CellProfiler. Color images were first converted into grayscale images using the modules "Color to gray" and "Invert for printing". Next, grayscale images were used as an input for the module "Identify Primary Objects" to identify a bright object (organoid) of a diameter ranging 300-1200 px on a dark background. The properties of identified objects could then be measured using the module "Measure Object Size Shape". Undesired identified objects (such as debris) were filtered out using the "Filter Objects" module, based on their compactness, with a maximum value of 4. After filtering, the module "Measure Object Size Shape" was again used to measure the properties of the filtered identified objects (organoid). The results of this module were exported to Excel file for analysis. The organoid area measured at day 2 was used as a reference starting point and therefore subtracted from

each individual value obtained. thereafter. At least 5 organoids per cell line for each time point from 3 independent experiments were analyzed. For the viability assay, control MO were treated from day 2 to day 11 every second day with different concentrations (0, 1-1-5 and 10 μ M) of Talarozole or Sertaconazole. DMSO control samples contained 0.1 % (v/v) DMSO, which is equivalent to the amount of solvent in the samples that received compound treatment. On day 9, organoid viability was measured using CellTiter-Glo cell viability assay following a previously published protocol.¹⁹ Briefly, the media of each well was adjusted to 55 μ l and the plates with MO together with the kit's reagents were brought to RT for 30 min. Next, an equal volume of 55 μ l of Celltiter-Glo reagent was added and samples were shaken on a Thermomixer at 900 rpm for 5 min. Afterwards, plates were incubated for 25 min at room temperature (RT) protected from light. The contents of the plate were transferred to white/opaque 96-well plates with 2 technical replicates (20 μ l each) per sample. Luminiscence of each sample was recorded using a Tecan plate reader. Extracellular lactate released by MO in the media was measured by collecting 100 μ l of maturation media at day 9 of MO generation. Media from at least 5 individual MO from 3 different batches was collected for analysis and stored at -20 °C until used. The manufacturer's instruction from the Lactate Assay commercial kit was followed for measuring the absorbance of each sample using a clear 96-well microplate assay and Tecan microplate to read the absorbance at 580 nm. The calculated amount of lactate per well was normalized to the respective organoid size (see *MO growth rate analysis* section) and expressed as fold-change compared to control (when untreated) or to DMSO-treated sample (when treated with Sertaconazole and Talarozole) of their respective batch.

Single-cell RNA sequencing (scRNAseq) analyses

35 days-old MO from iPSC control line were dissociated for scRNAseq using the Papain Dissociation System. Papain was prepared by adding 5 mL of EBSS to one papain vial and incubating for 10 min at 37 °C. Additionally, 500 μ l of EBSS was added to one DNase vial and gently mix. Half of the volume of DNase in EBSS was then added to the papain vial resulting in a final concentration of approximately 20 units/ml of papain and 0.005 % DNase. About 48 individual MO were collected onto a 6 well-plate, washed with PBS, and incubated with 2 mL of pre-warmed papain/DNase solution for 5-10 min at 37 °C in an orbital shaker. Using a 1 mL pipette, the organoids were gently triturated by mixing up and down and placed back into the incubator for another 5-10 min for further enzymatic digestion. The digested organoids were then collected in a 15 mL tube and 5 mL of ovalbumin was added. Using a 225 mm polished glass pipette, the mixture was further triturated until mostly dissociated. The cell suspension was then filtered through a 30 μ m cell strainer and transferred to a new 15 mL tube. The cell concentration was determined, and cells were pelleted by centrifuging

at 300 x g for 5 min. After removing the supernatant, the pellet was resuspended in 200 µl of ice-cold PBS and 800 µl of freezing-cold 100 % methanol was added. The cell suspension was snap-frozen in liquid nitrogen and stored at -80 °C until library preparation. After rehydration of the samples, libraries were generated using 10x Chromium and sequenced with Illumina NovaSeq 6000 platform. Samples were subjected to scRNAseq using the 10x Genomics Chromium Single Cell 3' Gene Expression system with feature barcoding technology for cell multiplexing. Demultiplexed, raw paired-end scRNAseq data was processed using the Cell Ranger (v. 7.10) from the 10x Genomics. Data was analyzed in three biological replicates, where each replicate was composed of 48 individual MO. Sequencing was carried out in two separate batches. In the first batch, replicates 1 and 2 were processed, while replicate 3 was processed in the second batch. In this manner, we assessed reproducibility of the sequencing results across different independent runs. For comparing our MO to other MO generated before, we employed scRNAseq datasets of MO that were previously generated at different time points.²¹ The datasets were obtained from GSE133894. Cell type was annotated following the approach described before¹⁹ where eight cell types were determined based on the expression levels of their marker genes (Endothelial cells, non-mDANs, mDANs, Glia, Nbs in vitro, NPCs and Pericytes). The percentage of each cell type with respect to the total cell counts was computed. Integration of the two datasets was performed using the *FindIntegrationAnchors()* function described before⁶⁵ with default parameters using the Seurat R package (v. 4.2.0).⁷⁰

scRNAseq data of Leigh cerebral organoids (CO) and isogenic control CO were carried out before.¹⁶ Datasets were obtained from GSE126360. Pseudotime analysis was performed on the RG, IPCs and Neurons populations in those scRNAseq data using Monocle3 R package.⁷¹ Input genes were differentially expressed genes among the three populations with the adjusted p value cutoff ≤ 0.05 . The DDRTree algorithm was used for the inference.

Immunostaining

Free-floating MO were fixed with 4 % PFA for 20 min at RT, followed by three 10 min washes with PBS. Organoids were blocked and permeabilized with a solution consisting of 3 % BSA, 0.5 % Triton-X-100 and 0.05 % sodium azide in PBS, for 1 h at RT. Primary antibodies were diluted in blocking solution and incubated overnight at 4 °C. Afterwards, they were washed 3 times with PBS and incubated overnight at 4 °C with secondary antibodies at a 1:1000 dilution in blocking solution. Next, organoids were incubated for 1 h at RT with Hoechst 33342 (1:1000) and washed three times with PBS. Images were acquired using the ZEISS Axio Observer microscope with an apotome 3 as a z-stack, which were then deconvoluted using the Zeiss blue software default settings and z-projected with maximum intensity. Antibodies are reported in the **Key Resource Table**. For quantification of tyrosine

hydroxylase (TH) within MO, immunostained MO z-projected images were analyzed using Columbus software (v. 2.9.0). Prior to analysis, images were downsampled using the greyscale function in Photoshop (v. 12.1 x64) and resized from 300 to 72 pixels per inch. The images were converted from 8-bit to 16-bit channels to retain image information and saved as .tif files. An image analysis pipeline was established using different building blocks. MO objects were identified based on the TH channel using the "Find Image Region" block. The "Fill Region" and "Border" functions within the "Select Region" block were utilized to cover the entire organoid area. Within this defined area, the intensities of each channel were calculated using the "Calculate Intensity Properties" block and given as the average pixel intensity. Furthermore, morphological parameters such as area, width, length (all measured in μm), and roundness were assessed using the "Calculate Morphology Properties" block. For the comparison of Leigh MO and control MO, we calculated the ratio of intensities of respective channels and the area of the individual MO.

Intracellular calcium imaging of MO

33-37 days-old MO were stained with the membrane-permeable form of the calcium indicator Fura-2 (Fura-2 AM; 1 mM stock solution in 20 % pluronic/DMSO; 15 μM final concentration) dissolved in artificial cerebrospinal fluid (ACSF), containing (in mM): 138 NaCl, 2.5 KCl, 2 CaCl_2 , 1 MgCl_2 , 1.25 NaH_2PO_4 , 18 NaHCO_3 , and 10 glucose (pH 7.4; osmolality ~ 310 mOsm/l) at 37 °C in a humidified atmosphere with 95 % O_2 and 5 % CO_2 for 30 min, followed by additional 30 min in ACSF without dye. A single MO was placed onto an Omnipore membrane filter and stabilized by a grid in the recording chamber. MO were continuously superfused (2–2.5 ml/min) with ACSF bubbled with 95 % O_2 5 % CO_2 and allowed to equilibrate for ~ 10 min before starting recordings. Experiments were carried out at 37 ± 1 °C. Wide-field calcium imaging was performed using an upright microscope equipped with a 40 x/N.A. 0.8 water immersion objective. Fura-2 AM was alternately excited at 380 and 400 nm using a monochromator and images were acquired at a frequency of 1 Hz with a complementary metal–oxide–semiconductor (CMOS) camera. Fura-2 AM emission was collected at 510 ± 40 nm from regions of interest (ROIs) manually drawn around cell bodies. Background correction was performed as previously described.⁷² Spontaneous calcium activity was analyzed during a 5 min recording period, in which no other manipulation was performed. Cells with calcium signals $\geq 2\times$ standard deviation (SD) were considered active. Metabolic stress was induced by perfusing preparations with a glucose-free ACSF containing 2 mM 2-deoxyglucose and 5 mM sodium azide for 2 min. Changes in fluorescence were normalized to the baseline and are expressed as percentage change.

Deep learning (DL)-based drug prediction

To predict genes that need to be perturbed for converting one cell fate state into another desired state, a feedforward artificial neural network (FNN) model was trained using the datasets retrieved from ChemPert.⁴⁸ We chose ChemPert since it consists of non-cancer perturbation transcriptomics datasets, which were shown to be more suitable for non-cancer cell studies than cancer-based datasets such as CMAP⁴² and LINCS.⁴³ The input data for training the FNN model was composed of: 1) differentially expressed transcription factors (TFs) upon cell stimulation by single perturbagens (both chemical and with protein agents), 2) initial (unperturbed) gene expression state of the cell. The latter was included to take into account cell-type specificity, which plays a critical role in determining transcriptional responses. The training output was the known direct target genes of perturbagens, which are also available in ChemPert. In total, we used 82270 transcriptomics datasets in response to 2566 unique perturbagens across 167 non-cancer cell types for the model training. In the next step, the predicted target genes were compared to the known target genes of 5693 perturbagens and the optimal perturbagen combinations were computed by maximizing the number of true positive (predicted) target genes, while minimizing the number of false positive (unpredicted but targeted by perturbagens) target genes. The FNN model performance was optimized by iterative cross validation. In our previous CO scRNAseq data of Leigh CO and isogenic control CO,¹⁶ we identified cell clusters for SURF1-corrected NPCs and neurons. We then performed differential expression analysis between these two cell clusters and the DETFs. The gene expression data of the NPCs were fed to the FNN model to identify perturbagens that could induce and accelerate the neurogenesis of Leigh NPCs. The model predicted 27 chemical compounds, 9 of which were short-listed for further experimental evaluation based on the prior knowledge of their biological effects.

DL algorithm

A feedforward neural network (FNN) model was trained using the perturbation transcriptomics datasets retrieved from ChemPert⁴⁸. Signaling proteins and their molecular interaction data were obtained from the following sources: protein-protein interactions from iRefIndex,⁷³ signaling networks from OmniPath,⁷⁴ ReactomeFI,⁷⁵ KEGG,⁷⁶ NicheNet,⁷⁷ and ligand-receptor interactions.⁷⁷⁻⁸⁸ Transcription factors (TFs) were obtained from AnimalTFDB 3.0 (<http://bioinfo.life.hust.edu.cn/AnimalTFDB2/>).⁸⁹ The perturbagens from ChemPert were further filtered by removing the ones targeting apoptosis-related proteins and those not targeting any signaling protein. The FNN model training data consisted of: 1) DETFs between initial and end cell states of each perturbation transcriptomics dataset, 2) gene expression of initial cell state. The FNN model output was direct target proteins of perturbagens. The model algorithm consists of four major steps described below.

Step 1: Gene expression value of each signaling protein p_j in the initial cell state is transformed by

$$x_j = \sum_i^n e_i p_j$$

where e_i is the gene expression of each direct upstream neighbour protein defined in Step 1. After computing this value for all signaling proteins, the normalization was performed by:

$$z_j = \frac{x_j - \min(x)}{\max(x) - \min(x)}$$

where x is the entire set of transformed signaling protein values. Differentially up- and down-regulated TFs were labeled with values of 1 and -1, respectively. 0 was assigned to all non-DETFs. Perturbagens' effect on direct protein targets –activation, inhibition or unknown–, were labeled with 1, -1 and 2, respectively.

Step 2: The transformed signaling protein values and DETF values are concatenated into one vector and fed to the FNN model for training. The number of hidden layers and nodes were optimized and set to 3, and 1024, 512, 1024 for hidden layers 1, 2, 3, respectively. Batch normalization is applied after each layer to enhance model stability. Backpropagation is performed using the Adam optimizer with a learning rate set to 0.005 to optimize the model using the cross-entropy loss function. The softmax function is used for the activation function in the output layer. The training epoch number is set to 1000.

Step 3: Pathway enrichment analysis is carried out on predicted target signaling proteins using the Reactome database. Pathways with fewer than 3 or more than 500 signaling proteins are excluded before analysis. Subsequently, enriched pathways with adjusted p-value ≤ 0.05 and containing more than 3 predicted signaling proteins are retained for the next step.

Step 4: For each enriched pathway, predicted signaling proteins present in that pathway are compared to the direct target proteins of perturbagens to identify optimal perturbation combinations by iteratively maximizing the Jaccard index:

$$J(P_j, Q_i) = \frac{|P_j \cap Q_i|}{|P_j \cup Q_i|}$$

where P_j is predicted target proteins in pathway j and Q_i is target proteins of perturbation i . First, a “seed” perturbation is matched to the predicted signaling proteins and the Jaccard index is computed. Then the next perturbation is matched to the remaining untargeted predicted signaling proteins and the Jaccard index is re-computed and if the index is higher than the previous value, then the perturbation is retained. This process is repeated until no increase in the Jaccard index is observed for all perturbations. Note, when the effect of predicted proteins is “unknown”, these proteins are considered both activation and inhibition. Perturbations with the Jaccard index less than 0.1 are discarded.

Neuromorphogenesis screenings in iNs

To generate iNs, NGN2 lentiviruses were produced using HEK 293 cells seeded at a 70 % confluence in a 150 cm² dish in DMEM medium.⁹⁰ Once attached, the medium was refreshed with DMEM supplemented with 25 µM Chloroquine and cells were transfected following Lipofectamine 2000 manufacturer's protocol using the following plasmid mix: 8.1 µg pMD2.G, 12.2 µg pMDLg-pRRE, 5.4 µg pRSV-Rev, 5.4 µg FUW-M2-rtTA, 14.3 µg TetO-FUW-NGN2, 14.3 µg TetO-FUW-EGFP.^{91,92} The next day, the medium was exchanged with DMEM-F12 containing 10 µM sodium butyrate. The supernatant containing viral particles was collected 24 and 48 h later. To concentrate the virus particles, the supernatant was first centrifuged at 500 x g for 10 min to remove cells and debris and then mixed with 1 volume of cold Lenti-X concentrator to every 3 volumes of lentivirus-containing supernatant. This mixture was kept overnight at 4 °C and further centrifuged at 1,500 x g for 45 min at 4 °C. The obtained pellet containing the virus was diluted 1:100 in PBS. Virus aliquots were stored at -80 °C until further use. For neuronal morphogenesis screening, NPCs were seeded onto matrigel-coated 96-well black greiner-bio plates at a density of 40,000 cells/well in 100 µl of sm+ (see *iPSC-derived neural progenitor cells* section for media composition). The next day, the media was refreshed with sm+ supplemented with 4 µg/ml polybrene and 2.25 x 10⁶ transducing units of NGN2 lentivirus. After overnight incubation, NPCs were washed 3 times with PBS and the media was refreshed with sm+ containing 2 µg/ml of doxycycline to start neuronal induction (day 0). On day 3, medium was exchanged to NGN2 medium, consisting of Neurobasal, B27 supplement with vitamin A (0.5 x), NEAA (1 x), 2 mM Glutamine, MycoZap-Plus-CL (1 x), 10 ng/ml human NT-3, 10 ng/ml human BDNF supplemented with 2 µg/ml doxycycline. On day 3, treatments with DLDs and YSDs were administered in the media using 3 different concentrations (1, 10, and 50 µM). Final concentration of DMSO in the media was either 0.1 % or 0.4 % (for 50 µM treatment of DLD4 and DLD5), which was equivalent to the amount of solvent in the samples that received compound treatment. On day 5, after 48 h incubation with the compounds, iNs were fixed by adding equal volumes of 8 % PFA to the medium. Cells were incubated in a final dilution of 4 % PFA for 20 min and washed 3 times with PBS for 10 min/wash. For staining, iNs were blocked and permeabilized for 1 h at RT with blocking solution (3 % BSA, Triton-X-100, 0.05 % sodium azide in PBS). Primary antibody incubation with anti-MAP2 (1:1000) in blocking solution was carried out overnight at 4 °C. Afterwards, they were washed 3 times with PBS and treated with Alexa Fluor Anti-Guinea pig AF568 secondary antibody (1:1000) and Hoechst 33342 (1:2500) in blocking solution for 1 h at RT. Next, after 3 washes with PBS, wells were filled with 0.05 % sodium azide in PBS and stored at 4 °C wrapped with parafilm until imaged. Antibodies are reported in the **Key Resource Table**. iNs were imaged with the high-content microscope

Operetta using the 0.4 NA 20X air objective. For each well, 20 areas across the well were selected for imaging. The areas remain constant for each plate analyzed. Images of Hoechst and MAP2 channel were then analyzed with CellProfiler⁹³ to analyze neuronal morphogenesis as previously described¹⁸ Data was summarized by calculating the mean per well. To compare different plates, numbers were compared to the mean of the DMSO-treated wells.

Yeast drug screen

Wild-type (WT) and SURF1-KO mutant (Δ SHY) strains were assessed for growth deficiency in a non-fermentable growth medium (YP 2 % lactate) at 30 °C. A 384-well optimization assay was developed: 1) to identify the right starting density of cells that accentuates the growth difference phenotype and best assay time, 2) to establish that the assay has a good Z', which is a measure of assay quality and the likelihood of false positives and negatives in the screen. In this assay, the Z' score was 0.7 indicating that the assay quality is excellent. Overnight cultures were diluted to an OD of 0.025. 50 nl of 10 mM test compounds were added to columns 3-22 of 384-well plates, while 50 nl of DMSO was added to control columns 1-2 and 23-24. Mutant and WT cell suspensions were adjusted to an OD of 0.025, and 25 μ l of mutant cells were added to columns 1-22, and WT cells to columns 23-24. The plates were spun at 250 x g for 1 min, sealed with a gas-permeable seal, and covered with a water-filled lid, then incubated at 30 °C for 24 h. After incubation, seals were removed and plates were equilibrated at RT for 15 min. 25 μ l of Bactiter-glo solution was added to each well, plates were shaken for 2 min, incubated for 10 min, and luminescence was read using an Envision plate reader. A list of hits was generated, with the top 2 % selected for further analysis. Test compounds used in yeast drug screening belong to the UCSF's Pharmakon library, comprising 1,600 drugs, and an additional 200 unique bioactives. All compounds are FDA-approved with an existing safety record.

Functional metabolomics based on CO scRNAseq

For the functional metabolic analysis of Leigh CO compared to isogenic control CO, we employed the Quantitative System Metabolism (QSM) pipeline developed by Doppelganger Biosystem GmbH, as described before.⁹⁴ QSM data analysis used quantitative information on the expression levels of metabolic proteins (enzymes) to determine metabolic profiles, metabolic states and capacities, and metabolic fluxes. The kinetic model includes cellular metabolic pathways related to energy metabolism, electrophysiological processes, and membrane polarization information, including the mitochondrial membrane potential membrane, the utilization of the proton motive force, and the transport of ions across the membranes. Time-variations of small ions were modelled by kinetic equations of the

Goldman-Hodgkin-Katz type.⁹⁵ Numerical values for kinetic parameters of the enzymatic rate laws were taken from reported kinetic studies of the isolated enzyme. Maximal enzyme activities (Vmax values) were estimated based on functional characteristics and metabolite concentrations of a healthy tissue. Hormone-dependent regulation of the central energy metabolism by reversible enzyme phosphorylation was taken into account by a phenomenological model of insulin and epinephrine dependent changes of plasma glucose and changes in the phosphorylation state of interconvertible enzymes as described in.⁹⁶ Expression profiles normalized to healthy neuronal tissue were used to scale the maximal activities of enzymes and transporters for each cell. QSM was used to calculate energetic capacity at maximal level at physiological nutrient and oxygen concentrations. The interdependence between plasma glucose, plasma hormone and plasma fatty acid concentration was taken into account by using a sigmoid Hill-type function describing the experimentally determined glucose-insulin and glucose-fatty acid relations.⁹⁷ Energetic capacities were evaluated by computing the changes of metabolic state elicited by an increase of the ATP consumption rate above the resting value.

Generation of midbrain dopaminergic neurons (mDANs)

The generation of mDANs from NPCs was performed following a previously published protocol.^{16,98} To start differentiation, NPC media was switched to a mixture of 1:1 Neurobasal:DMEM-F12, N2 (0.5 x), B27 with vitamin A (0.5 x), 2 mM glutamine and Mycozap-Plus-CL (1 x) supplemented with 200 µM ascorbic acid, 100 ng/ml FGF8 and 0.5 µM Purmorphamine for 7 days. The following two days, the concentration of ascorbic acid and purmorphamine was brought down to 100 µM and 0.25 µM, respectively. On day 9, cells were passaged with accutase and seeded onto matrigel-coated plates with 10 µM ROCK inhibitor in maturation media, made of a mixture 1:1 Neurobasal:DMEM-F12, N2 (0.5 x), B27 with vitamin A (0.5 x), 2 mM glutamine, Mycozap-Plus-CL (1 x), with the addition of 200 µM ascorbic acid, 10 ng/ml BDNF, 10 ng/ml GDNF, 500 µM dibutyryl-cAMP, and 1 ng/ml of TGF-β3. The media was refreshed every other day, and the neurons were kept in culture for 4 and 8 weeks to reach different maturation stages.

Mitochondrial movement in mDANs

mDANs cultures were grown on 35 mm dishes with coated bottom and 1.5 coverslips. 25 nM MitoTrackerRed CMXRos was added for 10 min and then replaced with DA culture media. Live-cell imaging recordings were conducted using a spinning disk microscope with incubating conditions of 37 °C with 5 % CO₂. Cells were imaged every 2 sec using a 40 x oil objective. The raw image files were stored as 16-bit in “.nd2” format at 337x337 µm (1024x1024 pixel) at an interval of 2 sec and a total of 200 images per series (total time per

series: 6:40 min). The image pre-processing was carried out with Fiji (ImageJ 1.52h) adapted from a previous publication.⁹⁹ To compensate for photobleaching during the time series, the “Bleach Correction” tool was applied, followed by a top-hat spatial filter to increase gray values of mitochondrial objects. The total mitochondria count was calculated by creating a binary image where the minimum gray value (min) was set to $\text{min} = \text{Mean} + \text{StdDev}$ and the maximum gray value (max) was set to $\text{max} = \text{Mean} + \text{StdDev} + 1$, where “Mean” and “StdDev” were obtained by using the “Measure” tool. Next, the “watershed” tool was applied to break up large mitochondria networks. Total number of mitochondria was quantified using the “Analyze Particles” tool with standard settings and a size preference for 8-200 pixel. The moving mitochondria were defined by a particle size of at least 6 pixels that changed location over the time course of 4 frames (=8 seconds). This was accomplished by subtracting the following 4th frame for each frame in the time series (on the top-hat filtered image). Afterward, the subtracted image series was converted into a binary image series. The number of moving mitochondria was similarly counted using the “Analyze Particles” tool with standard settings and a size preference for 6-200 pixel. Finally, the percentage of moving mitochondria per sample was calculated by dividing the average number of moving mitochondria by the average number of total mitochondria, multiplied by 100. The percentage of stationary mitochondria was obtained by the subtraction of moving mitochondria from 100.

Western blotting

For western blots, 50-90 individual MO chronically treated with either DMSO (0.1 % v/v), 0.1 μM Sertaconazole or 1 μM Talarozole were collected on day 28-30 of differentiation in a 1.5 ml tube. MO were washed 3 times with PBS and pelleted using a tabletop centrifuge at 8,000 rpm for 5 min. Fresh pellets were weighed and resuspended in appropriate volume of RIPA Lysis Buffer supplemented with proteinase inhibitor and phosphatase inhibitor (1:100) cocktails. Samples were then transferred to tubes containing CK14 ceramic beads and processed using a tissue homogenizer. The suspension was incubated on ice for 30 min, followed by centrifugation for 10 min at 10,000 rcf at 4 °C. Protein levels in the supernatant were then quantified using the BCA Protein Assay Reagent Kit. Protein lysates (35 μg) were separated on 4–12 % Bis–Tris gels, transferred to PVDF membranes, which were blocked with 3 % skimmed milk powder in TBS-T and incubated with antibodies against SNPH, KIF5A and ACTB as a housekeeping protein (see **Key Resource table** for full list of antibodies). After overnight incubation at 4 °C, membranes were washed and incubated with secondary antibodies horseradish peroxidase-conjugated anti-rabbit or anti-mouse. Signals were visualized using ECL solutions and ChemiDoc Touch Imaging System and the intensity of the bands was quantified using Image Lab software (v. 6.1.0). The intensity of the bands

of interest (SNPH and KIF5A) were normalized to ACTB content in the samples. Values of treated MO are expressed relative to DMSO-treated Leigh MO.

Targeted metabolomics

For targeted metabolomics analysis, NPCs were grown in Matrigel-coated 6-well plates until reaching 70 % confluence. NPCs were either left untreated or were treated with 0.1 % (v/v) DMSO, 10 μ M Sertaconazole, or 10 μ M Talarozole for 24 h. At this point, accutase was used to detach the cells from the plate. Cells were then centrifuged at 200 x g for 3 min and the obtained pellets were dissolved in PBS to allow for quantification of cells using an automatic cell counter. Using a gating size of 5-19 μ m, 1 million NPCs/sample were pelleted in a 1.5 ml tube using a tabletop centrifuge at 8,000 rpm for 5 min. The supernatant was discarded, and the pellet was snap-frozen in liquid nitrogen for subsequent measurement. Extraction of the targeted metabolites was performed by adding 300 μ l Methanol/H₂O (80/20; v/v) to the 1 x 10⁶ cells, which were homogenized by ceramic beads CK14 using Precellys lysing kit. The corresponding isotopically labelled internal standards were added to the extraction solution. The targeted compounds were analyzed by UPLC-MS/MS. The system consists of a UPLC I-Class (Waters) coupled to a tandem mass spectrometer Xevo-TQ-XS (Waters). Electrospray ionization was performed in the negative ionization mode for the compounds fructose-1,6-bisphosphate, phosphoenolpyruvate (PEP) and 2/3-phosphoglycerate.¹⁰⁰ The TCA, energy metabolites (AMP, ADP and ATP) as well acetyl- and succinyl-CoA were measured in the positive ionization mode.¹⁰¹⁻¹⁰³ Mass spectrometric quantitation of the compounds was carried out in the multiple reaction monitoring (MRM) mode. MassLynx software (v. 4.2) was used for instrument's control and data acquisition. Quantitation analysis was performed by TargetLynx XS software.

Cholesterol measurements

For cholesterol analysis, 40,000 NPCs per well were seeded onto a matrigel-coated 96-well black ibidi plates. The next day, NPCs were either fed with normal media (untreated condition) or treated with either 0.1 % (v/v) DMSO, 10 μ M Sertaconazole, or 10 μ M Talarozole for 48 h. For the 24 h treatment, NPCs were treated with the above-mentioned conditions two days after. Both 24 h and 48 h treatment conditions were fixed on the same day using 4% PFA for 15 min at RT and washed 3 times for 10 min with PBS. For PFO-GST labelling of membrane-bound cholesterol, fixed NPCs were blocked and permeabilized with 10 % goat serum in 0.1% Triton X-100, PBS (0.1 % PBST) for 1h and incubated with 15 μ g/ml recombinant PFO-GST for 3 h.¹⁰⁴ After washing the cells three times with 0.1 % PBST, anti-GST (1:200) was applied in 10% goat serum 0.1% PBST, overnight at 4 °C. After three washes with 0.1 % PBST, Alexa Fluor™ 488 goat anti-mouse IgG (H+L) secondary antibody

was applied (1:500) for 1 h at RT. Lastly, wells were washed three times in PBS and cells were preserved in Mounting Medium with DAPI. For neutral lipid labelling, fixed cells were incubated with 1 µg/mL BODIPYTM 493/503 in PBS for 20 min, followed by three washes of 5 min with PBS and mounting medium with DAPI was added to the wells. Fluorescent images were acquired with a LSM 900 Zeiss confocal microscope. Laser power, gain and offset parameters were kept constant for each experiment, any subsequent adjustments to contrast and brilliance were applied equally to all images. The image analysis was performed using Fiji ImageJ software using custom-made macros.

Molecular docking

To test Sertaconazole and Talarozole binding to postulated targets, we performed *in silico* docking experiments. We modeled docking to the protein target of Talarozole cytochrome P45026 (CYP26A1/B1). Since there is no experimental structure reported to date for CYP26A1/B1, we obtained CYP26A1/B1 from the alphafold2¹⁰⁵ database based on PDB:2VE3 which reports the structure for retinoic acid bound to cyanobacterial CYP120A1. Overlap between the two protein structures enabled the localization of the iron-heme and the substrate structure within the cavity of CYP26A1/B1.¹⁰⁶ Removing the substrate and redocking it together with Sertaconazole and Talarozole revealed difference in poses and docking scores. CCDC Gold v2022 was used for docking and CCG MOE (v. 2022.02) was used to visualize the results and generate images. Compound poses were analyzed using docking scores and by the minimal distance of any compound's atom from the heme-iron reactive center for oxidation. The combination of distance from the iron atom and the relative energy needed to reach the ligand's position can suggest whether compounds act as substrate or as inhibitor, since inhibitors are usually closer to the iron-center disturbing the oxygen recruitment from heme.^{107,108}

PCR and gene expression analyses

For quantitative real-time PCR (qPCR) analysis of CYP26B1 in iNs, 2 million NPCs were seeded onto matrigel-coated 6 well-plates one day prior to NGN2 lentiviral transfection supplemented with 4 µg/ml polybrene. After overnight incubation with NGN2 lentiviruses, cells were washed 3 times with PBS and the media was refreshed with sm+ containing 2 µg/ml of doxycycline to start neuronal induction (day 0). Three days later, media was exchanged to iN media (see *Neuromorphogenesis analysis in iNs* for media formulation) with either DMSO (0.1 % v/v), 10 µM Sertaconazole, or 10 µM Talarozole. On day 5, treatment was refreshed to incubate for another 6 h to observe changes in gene expression.¹⁰⁹ Afterwards, cells were collected using a cell scraper, pelleted using a tabletop centrifuge at 8,000 rpm for 5 min, and snap-frozen in liquid nitrogen. RNA extraction from pellets was

performed with RNeasy kit. cDNA was generated from 2 µg RNA using the first strand cDNA synthesis kit. qPCR experiments were conducted for three technical replicates and three biological iNs replicates with the SYBR green Mastermix and CFX96 Real-Time System (Bio-Rad). After averaging the CT values of the three technical replicates, the 2-ΔΔCT method was used. The expression levels of the genes of interest were normalized relative to the average expression of housekeeping genes (C1orf43 and YWHAZ) and relative to DMSO treatment. Primer sequences are reported in the **Key Resource Table**.¹¹⁰ Expression of anchoring and RA pathway genes was obtained from our previously published bulk RNAseq datasets for Leigh mDANs and Leigh CO.¹⁴

QUANTIFICATION AND STATISTICAL ANALYSIS

Data are expressed as mean and standard deviation (mean ± SD) where normality of the distribution could be verified, or as median and quartiles (median [1st, 4th quartiles]) otherwise. Significance was assessed using parametric tests (Welch's t-test, ANOVA) for normally distributed data and non-parametric tests (Mann-Whitney U test, Kruskal-Wallis) when normal distribution could not be verified. Unless otherwise indicated, data were analyzed and shown using GraphPad-Prism software (v. 10.2.2) (GraphPad Software, USA) or R. Schematic cartoons were made using Biorender.

Figure Legends

Figure 1. Derivation of 2D and 3D pipelines for targeting neuromorphogenesis in Leigh. (A) Schematic view of the 2D and 3D pipelines to assess the ability of NPCs to acquire neuronal fate. (B) Outgrowth capacity in induced neurons (iNs) from Leigh and isogenic control (CTL) based on high-content analysis (HCA) quantification. Mean + SD with dots representing 12 biological replicates over 2 independent experiments; * $p < 0.05$, unpaired two-tailed t test. Scale bars: 100 μm . (C-D) Unsupervised clustering and population distribution of scRNAseq of our midbrain organoids (MO) ("This paper") compared to previously derived MO ("Zagare et al"). (E) Leigh MO growth rate. Dots represent individual MO over 3 independent experiments; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$, two-way ANOVA. (F) Lactate release from MO. Dots represent individual MO over 3 independent experiments. **** $p < 0.001$, Mann-Whitney U test. (G) Calcium response in MO following the application of metabolic stress (m.s.) for 2 minutes. Dots represent individual cells (7 CTL MO and 6 Leigh MO) over 2 independent experiments; **** $p < 0.001$, Mann-Whitney U test. (H-J) Organization of dendrites (MAP2-positive) and axons (SMI312-positive) and amount of tyrosine hydroxylase (TH)-positive cells in MO. Dots represent individual MO over 3 independent experiments; ** $p < 0.01$, unpaired two-tailed t test. Scale bars: 100 μm .

Figure 2. Neuromorphogenesis screenings in Leigh models identified Talarozole and Sertaconazole. (A-B) UMAP plots of scRNAseq of cerebral organoids (CO) from Leigh and isogenic control (CTL) showing uncommitted progenitors with radial glia (RG) identity and neural committed cells with identity of intermediate progenitor cells (IPCs) and mature neurons (Neurons). (C-D) Pseudotime analysis of the three populations (RG, IPCs, and Neurons). (E-F) Schematic of the developed deep learning (DL) algorithm to identify drugs capable of promoting differentially expressed transcription factors (DETF)-specific cell fate conversion. (G) Schematic of the 2D neuromorphogenesis screening in Leigh iNs using DL-predicted drugs (DLDs) and yeast screen drugs (YSDs). (H-K) DLDs and YSDs impact on neurite outgrowth and representative images for DLD8 (Talarozole) and YSD4 (Sertaconazole). Mean + SD of 5 biological replicates (dots); Statistics is shown only for ameliorating compounds, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$, unpaired two-tailed t test, compound-treated Leigh iNs vs DMSO-treated Leigh iNs. Scale bars: 100 μm .

Figure 3. Sertaconazole and Talarozole improve neuromorphogenesis in Leigh organoids without impacting mitochondrial anchoring. (A) Schematic of treatment in Leigh midbrain organoids (MO). (B) Average growth rate profile of isogenic control (CTL) MO

is shown with dotted gray lines. Dots represent individual MO over 3 independent experiments; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$, two-way ANOVA, compound-treated Leigh MO vs DMSO-treated Leigh MO. **(C)** Lactate released in treated Leigh MO compared to DMSO-treated Leigh MO. Dots represent individual MO over 3 independent experiments. ** $p < 0.01$, **** $p < 0.001$, Mann-Whitney U test, compound-treated Leigh MO vs DMSO-treated Leigh MO. **(D-E)** Neurite organization and quantification of tyrosine hydroxylase (TH)-positive neurons in treated Leigh MO. Dots represent individual MO over 3 independent experiments; * $p < 0.05$, unpaired two-tailed t test, compound-treated Leigh MO vs DMSO-treated Leigh MO. Scale bars: 100 μm . **(F)** Mitochondrial motility in midbrain dopaminergic neurons (mDANs) from Leigh and isogenic control (CTL) at 4 weeks (4w) or 8 weeks (8w) of differentiation starting from NPCs. Dots represent individual mDANs over 3 independent experiments; *** $p < 0.005$; unpaired two-tailed t test, Leigh mDANs vs CTL mDANs. Scale bars: 100 μm . **(G-H)** Representative immunoblot and related quantification of mitochondrial anchoring proteins SNPH and KIF5A in CTL MO, Leigh MO, and treated Leigh MO. The experiments were performed in 3 independent experiments (dots).

Figure 4. Sertaconazole and Talarozole modulate cholesterol/energy metabolism and retinoic acid (RA) signaling in Leigh models. (A-E) Functional metabolic analysis inferred from scRNAseq of cerebral organoids (CO) from Leigh and isogenic control (CTL). Dots represent individual cell values for 3 independent CO scRNAseq samples. ** $p < 0.005$; unpaired two-tailed t test. **(F-H)** Targeted metabolomics in NPCs. Dots represent biological replicates over 2 independent experiments; ns: not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$; unpaired two-tailed t test. **(I-K)** Membrane-bound cholesterol levels measured by PFO staining in CTL NPCs, Leigh NPCs, and treated Leigh NPCs. Dots represent individual values over 3 independent experiments; *** $p < 0.005$; unpaired two-tailed t test. Scale bars: 100 μm . **(L-N)** Predicted docking ability for Sertaconazole and Talarozole to bind the CYP120 pocket. **(O)** Expression of retinoic acid (RA)-related genes in Leigh mDANs compared to CTL mDANs. * $p < 0.01$, fold change expression from bulk RNAseq dataset. **(P)** CYP26 mRNA expression in Leigh induced neurons (iNs) treated with Sertaconazole or Talarozole compared to DMSO-treated Leigh iNs. *** $p < 0.005$; unpaired two-tailed t test. **(Q)** Schematic of proposed mechanisms of action of Sertaconazole and Talarozole in Leigh.

Figure S1. Neuromorphogenesis defects in 2D and 3D models of Leigh (related to Figure 1). (A-B) Neurite outgrowth defects in Leigh iNs after NGN2 induction based on GFP signal that is part of the NGN2 inducible cassette. Mean + SD, dots represent 12 biological replicates over 2 independent experiments. Scale bars: 100 μm . **(C)** Schematic of midbrain organoid (MO) protocol. **(D)** Day 30 MO contained TH and NURR1-positive midbrain

dopaminergic neurons (mDANs). Scale bars: 100 μ m. **(E-K)** Functional calcium imaging in Leigh MO and CTL MO. Dots represent individual cells in MO (7 CTL MO and 6 Leigh MO) over 2 independent experiments; ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$, Mann-Whitney U test.

Figure S2. Neuromorphogenesis screenings in Leigh models with DL-predicted drugs (DLDs) and yeast screen drugs (YSDs) (related to Figure 2). **(A)** UMAP showing unbiased clustering of CO scRNAseq. **(B)** Percent distribution of radial glia (RG) (cluster 7), intermediate progenitor cells (IPCs) (cluster 4) and neurons (cluster 6) at different pseudotime states in CO from Leigh and CTL. **(C)** Schematic of DL strategy. **(D-E)** Neuromorphogenesis screen in Leigh iNs based on HCA quantification of neurite outgrowth. Mean + SD of 5 biological replicates (dots); ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$, unpaired two-tailed t test, compound-treated Leigh iNs vs DMSO-treated Leigh iNs. **(F-H)** Viability assay in cultures from wild-type (WT) yeast and from yeast knock-out for the SURF1 homologue SHY1 (Δ SHY). **(I)** FDA-approved drugs screened in Δ SHY yeasts. Dots represent individual drug response; green dots: top 50 yeast screen drugs (YSDs); purple dots: YSD selected for validations in Leigh iNs. **(J-K)** Neuromorphogenesis screen in Leigh iNs based on HCA quantification of neurite outgrowth. Mean + SD of 5 biological replicates (dots); ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$, unpaired two-tailed t test, compound-treated Leigh iNs vs DMSO-treated Leigh iNs.

Figure S3. Energy metabolism and mitochondrial anchoring in Leigh models (related to Figure 3 and Figure 4). **(A-B)** Viability experiments in CTL MO showed that 0.1 μ M Sertaconazole and 1 μ M Talarzole were the highest concentrations that could be used for long-term treatment of MO. Dots represent individual MO over 3 independent experiments; **** $p < 0.001$, unpaired two-tailed t test. **(C)** Representative images used to establish the pipeline to quantify moving and stationary mitochondria within midbrain dopaminergic neurons (mDANs) based on live cell tracking with MitoTracker Red. **(D)** Mitochondrial motility in CTL mDANs and Leigh mDANs upon acute exposure to the mitochondrial uncoupler FCCP. Dots represent individual mDANs over 3 independent experiments; *** $p < 0.005$; unpaired two-tailed t test. **(E)** Left: Schematic depicting the relevance of anchoring proteins for mitochondrial motility. Right: expression of anchoring genes in Leigh mDANs and Leigh CO compared to CTL. * $p < 0.01$, fold change expression from bulk RNAseq dataset. **(F-G)** tSNE plot of scRNAseq datasets from Leigh CO and CTL CO used for functional metabolic analysis showing different distribution of population with respect to all genes but overlapping profile for metabolism-related genes. **(H-K)** Functional metabolic changes in Leigh CO compared to CTL CO. Dots represent individual cell values for 3 independent CO scRNAseq samples.

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Figure S4. Mechanism of action studies in Leigh models treated with Sertaconazole or Talarozole (related to Figure 4). (A-D) Targeted metabolomics for ATP and glycolysis-related metabolites in CTL vs Leigh NPCs and DMSO-treated Leigh NPCs vs. compound-treated Leigh NPCs. Dots represent biological replicates over 2 independent experiments; ns: not significant, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$; unpaired two-tailed t test. (E-F) Targeted metabolomics for TCA cycle related metabolites in CTL NPCs vs Leigh NPCs and DMSO-treated Leigh NPCs vs. compound-treated Leigh NPCs. Dots represent biological replicates over 2 independent experiments; ns: not significant, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$; unpaired two-tailed t test. (G-H) Quantification of neutral lipids stained with BODIPY in CTL NPCs and Leigh NPCs treated with Sertaconazole or Talarozole. Dots represent individual NPC samples over 3 independent experiments; ns not significant, *** $p < 0.005$; unpaired two-tailed t test. Scale bars: 100 μm . (I-K) Docking modeling prediction for Sertaconazole and Talarozole with flexible configuration or with configuration fixed on the CYP120 pocket.

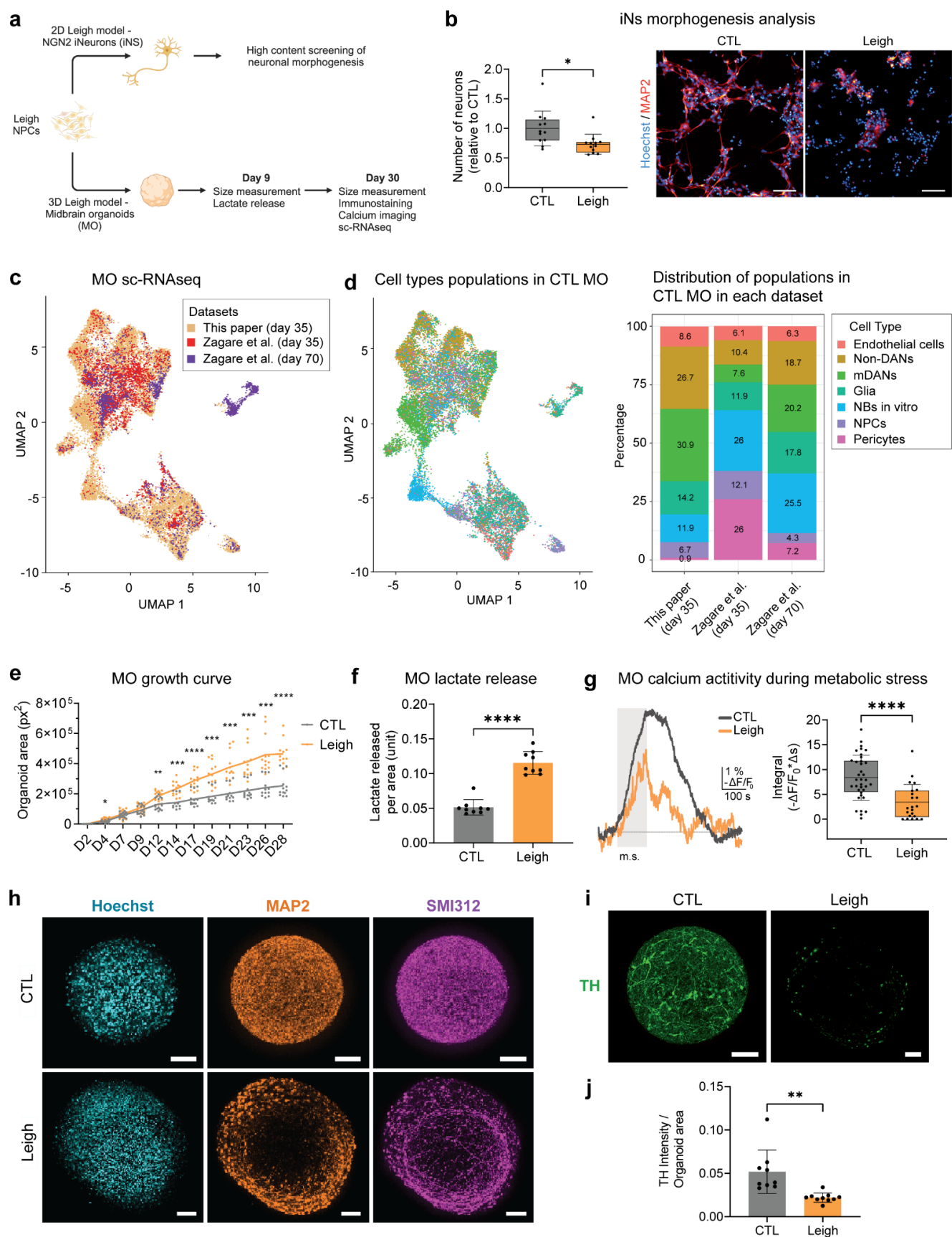
Figure1

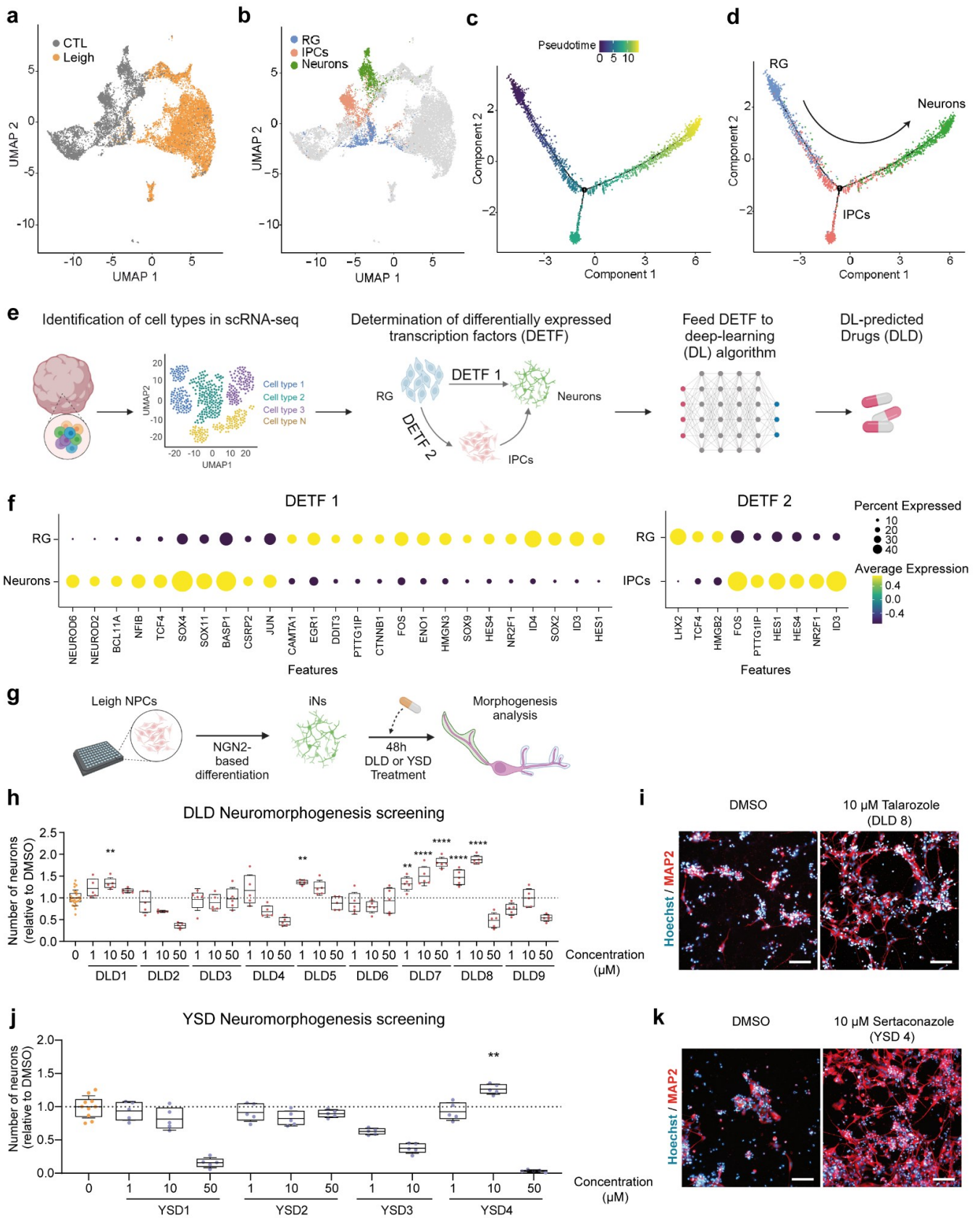
Figure2

Figure3

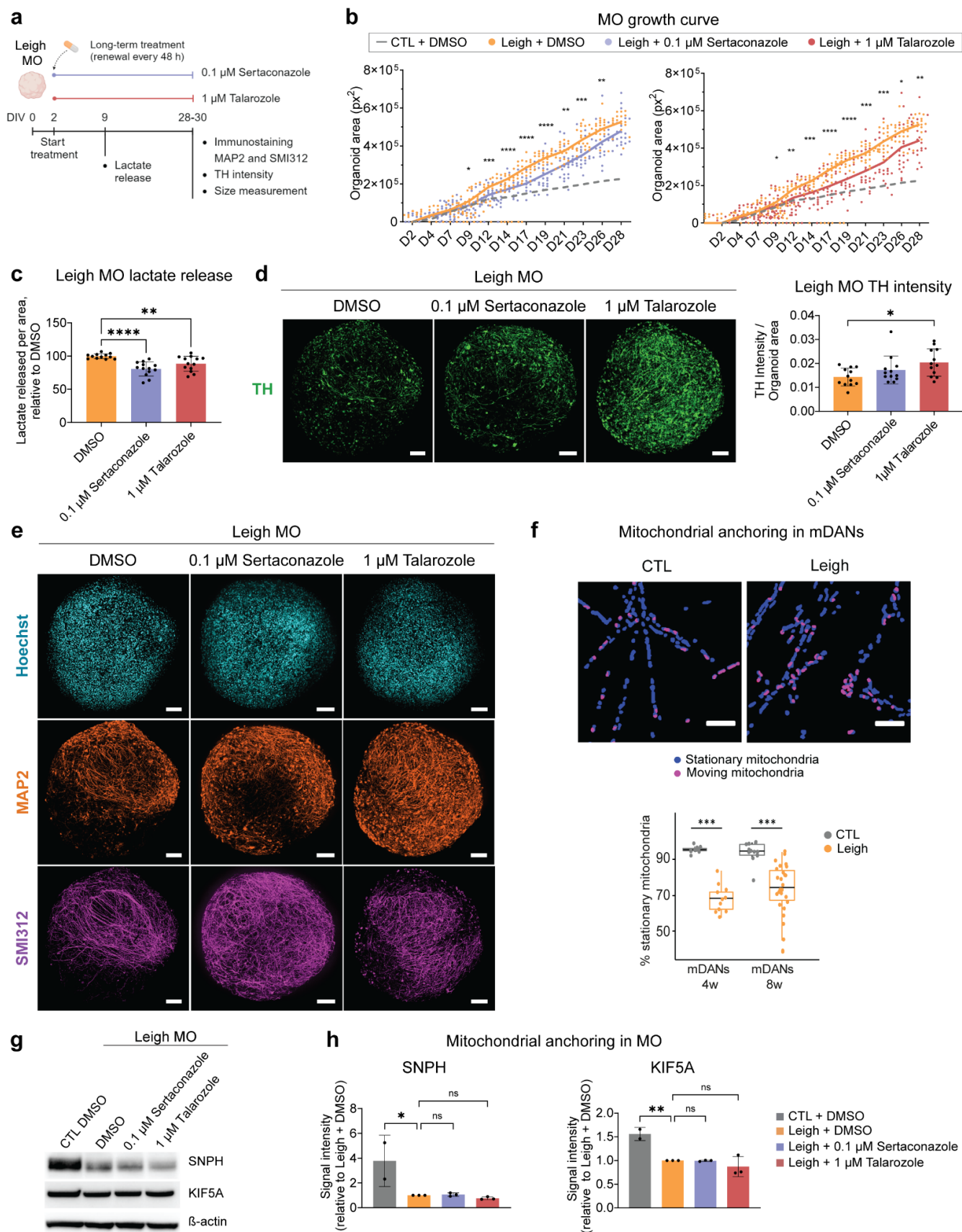
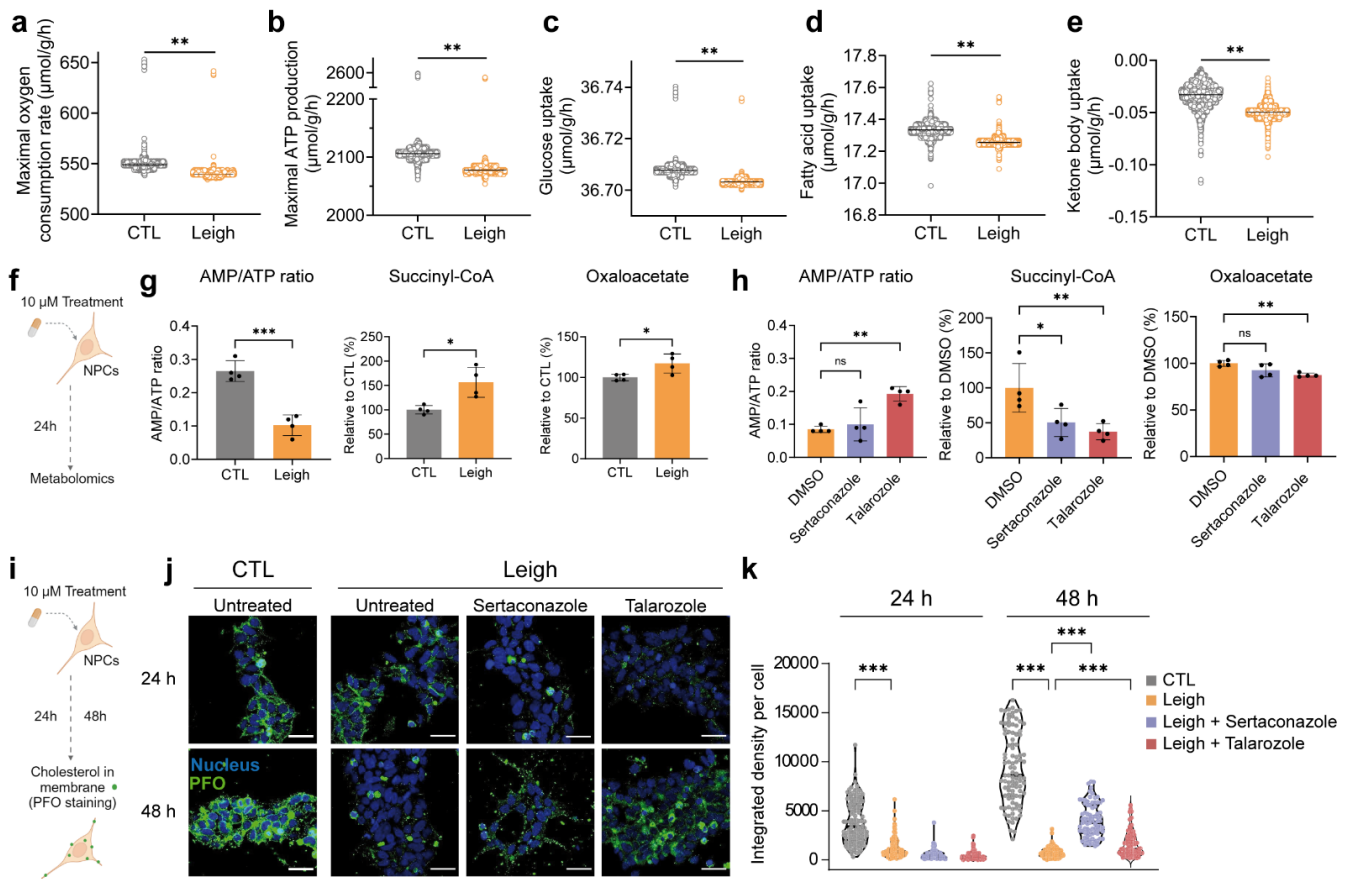
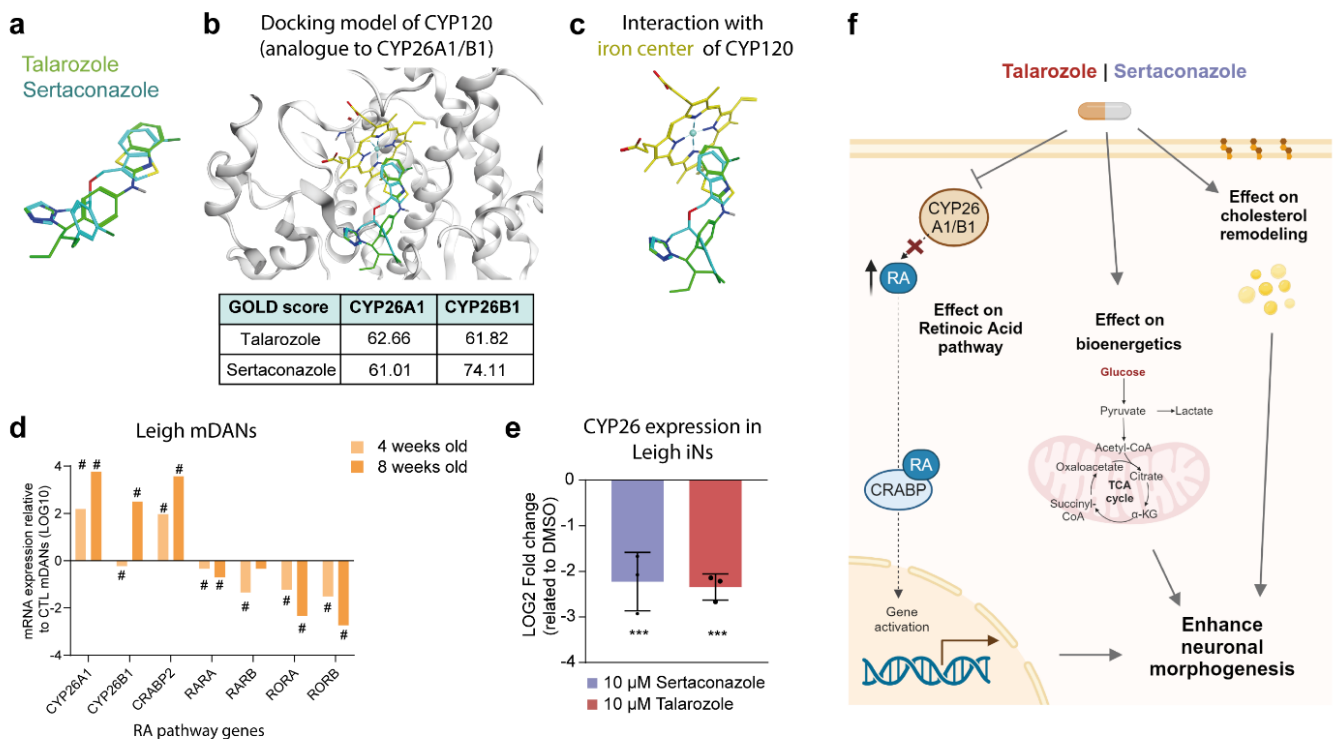
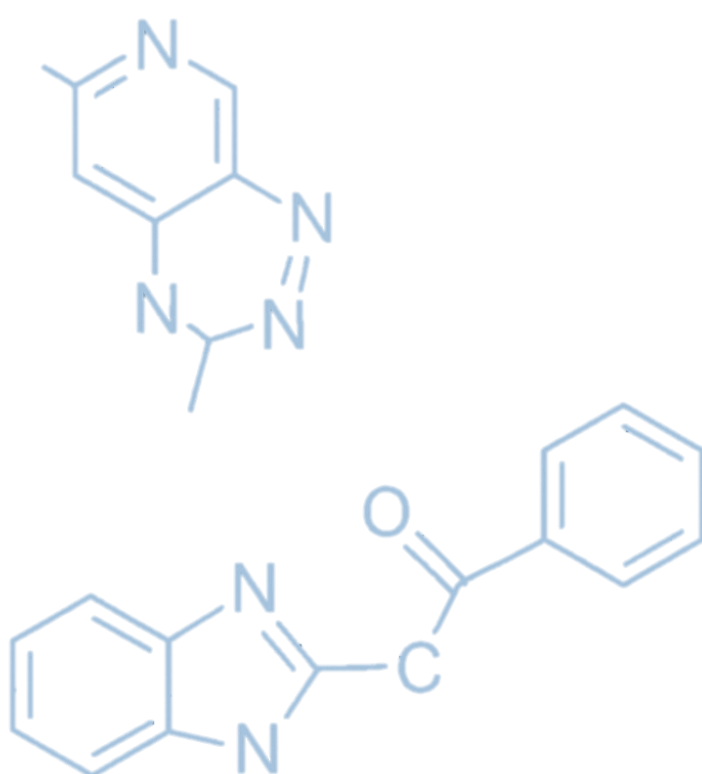


Figure 4**Figure 5**

4. DISCUSSION



The discussion of this thesis is divided into four sections.

4.1: DEVELOPMENT OF HIGH CONTENT IMAGED-BASED NEUROMORPHOGENESIS PIPELINE TO ASSESS DEFECTS IN LEIGH SYNDROME

This section discusses the development of the pipeline published by Lickfett S, et al 2022 and its application to identify neuromorphogenesis defects in LS, reported in Menacho et al., 2024 (in revision).

4.2: ESTABLISHMENT OF MIDBRAIN DOPAMINERGIC ORGANOIDs FOR DISEASE MODELLING AND DRUG DISCOVERY

This section evaluates the use of midbrain organoids as a disease-relevant 3D model to study defective neuronal morphogenesis linked to mitochondrial dysfunction in the context of HD (Lickfett et al., 2024) and LS (Menacho et al., 2024, in revision).

4.3: DRUG DISCOVERY PIPELINES IN LS IDENTIFY AZOLE COMPOUNDS

The full content of this section discusses the results submitted by Menacho et al., 2024 to Nature Communications (currently in revision) addressing the discovery of azole compounds for the treatment of LS phenotypes.

4.4: DECIPHERING THE MECHANISM OF ACTION OF AZOLE COMPOUNDS

This section evaluates the potential mechanism of action by which azole compounds ameliorate LS phenotypes and addresses the limitations of the results in Menacho et al., 2024 (in revision).

4.1. DEVELOPMENT OF HIGH CONTENT IMAGING-BASED NEUROMORPHOGENESIS PIPELINE TO ASSESS DEFECTS IN LS

4.1.1. Development of a high-content imaging analysis pipeline to assess neuronal morphogenesis

With the ultimate goal of performing phenotypic-based drug discovery for LS (Menacho et al., in revision), I first developed a pipeline to measure neuronal morphogenesis in iNs in a HCI manner (Lickfett et al., 2022). This semi-automated pipeline to measure neuronal morphology was developed using CellProfiler, a free, open-source software (Carpenter et al., 2006). This software is designed to automatically measure specific phenotypes from a large quantity of images using advanced algorithms available as individual modules that can be placed in sequential order to form a pipeline. Thus, CellProfiler allows the democratization of image analysis regardless of coding experience and without excessive costs.

Our published protocol provides an adaptable pipeline to measure neurite outgrowth by following the sequential process of (i) identifying cell nuclei using the “identify primary objects module”, (ii) identifying neurites (dendrites or axons, depending on the staining used) arising from those previously identified nuclei by employing the “identify secondary objects” module; and finally (iii) isolating neurites from the soma by using the “identify tertiary objects” module. These objects (neurites) can then be skeletonized to reduce their essential geometric features to quantify the total neurite length, number of trunks, number of branching points and number of endpoints (Lickfett et al., 2022).

The adaptable protocol follows the classic methodology used by most morphometric approaches. This methodology involves determining the nucleus and soma of the cell as the origin point to identify branches emanating from it (Haas et al., 2016). Traditionally, such neurite tracing has been performed manually to achieve the most accurate results, using programs such as Neurolucida (MicroBrightField) or IMARIS (Oxford instruments). Despite its accuracy, manual tracing of neurites is a laborious, highly time-consuming task best suited for smaller datasets rather than for high-throughput analysis.

Similarly, manual Sholl analysis has been the gold-standard method to quantitatively describe the degree of complexity of dendrite arborization in the field of neuronal morphometrics. Sholl analysis (Sholl, 1953) consists of drawing concentric spherical circles from the soma and counting the intersections of axons and dendrites at increasing distances from the soma. While informative, manual Sholl analysis requires sparse neuronal cultures and is a time-consuming process that limits the number of analyzable cells.

In disease modeling and drug discovery, HCI combined with semi-automated and automated algorithms enables rapid, unbiased quantification of neurite metrics. Private software for image analysis, such as CellInsight (ThermoFisher) and Columbus (PerkinElmer), offer user-friendly programs with specific features to ease neurite tracing. However, these systems often require extensive parameter optimization, resulting in high costs for the user, limiting their accessibility. Instead, free semi-automated ImageJ plugins such as NeuroJ (Meijering et al., 2004), NeuriteTracer (Pool et al., 2008), or SNT (Arshadi et al., 2021) are available to everyone. Recently, even semi-automated methods of the classically used Sholl analysis have become available (Ferreira et al., 2014; Schmuck et al., 2020). However, these require isolated and poorly connected neurons for proper identification, in addition to trained personnel with coding experience for analysis (Binley et al., 2014; Ferreira et al., 2014; Schmuck et al., 2020).

While CellProfiler provides a powerful tool for HCI analysis, the dynamic tracing of neurite outgrowth requires live-cell imaging approaches. Neuronal morphogenesis is a dynamic process characterized by extensive branch turnover, ultimately shaping the mature neuronal architecture. Fully understanding how neurons establish their complex arborization requires dynamic morphometric measurements to track structural changes over time.

Live-cell imaging techniques, such as time-lapse microscopy, combined with semi-automated software like Incucyte Neurotrack (Sartorius), offer a means to monitor neurite outgrowth in real time. While this system enables neurite tracing using phase-contrast imaging, fluorescence microscopy provides more reliable results, particularly in multicellular cultures or under experimental treatments. However, a major limitation of live-cell neuronal tracking is its inability to distinguish neuronal progenitors from more committed iPSC-derived neurons. Genetic labeling or the delivery of fluorescent plasmids could enhance cell-type specificity in these cultures. In our hands, however, lentiviral-mediated GFP expression did not yield optimal results for downstream analysis, as differences in proliferation rates between cell lines and aberrant marker distribution in patient-derived neurons introduced variability (not shown).

We selected CellProfiler as a free, semi-automated software capable of analyzing large datasets that allows the creation of adaptable pipelines for the fast and unbiased quantification of neurites in highly connected iPSC-derived neuronal cultures. Using this pipeline, we were able to assess the effect of genetic manipulation of selected genes in the context of Huntington's disease neuropathology (Lisowski et al., 2024) and I was able to determine neuromorphogenesis defects in *SURF1*-mutant iNs that were used as a phenotype for drug discovery (Menacho et al., in revision). Its adaptability makes the pipeline suitable for multiple experimental designs, from different differentiation protocols (small molecule-derived, lentiviral-based) to different starting points (iPSCs, NPCs) and different morphological readouts (outgrowth vs morphogenesis).

While CellProfiler provides a cost-effective and adaptable solution, it has its limitations. Its user interface may require a steeper learning curve compared to commercially available software. In addition, while its predefined modules ease the task of image analysis for people with little experience in the field, highly customized analysis may still require additional scripting using Python or MATLAB. Furthermore, while it succeeds at HCI analysis, it may encounter its limitations in distinguishing individual neurites in highly overlapping networks, which could introduce variability in experimental conditions.

Despite presenting these limitations, this pipeline could be adapted to other neurodevelopmental and neurodegenerative disorders, as well as be used for toxicity assays (Zink et al., 2020) or to assess the effect of gene manipulation in neurite branching (Lisowski et al., 2024).

Given the growing interest in phenotypic screening and personalized medicine, integrating this semi-automated approach with machine learning-based image analysis could further enhance its potential in drug discovery, leveraging the strengths of both methods. For instance, CellProfiler relies on thresholding and morphological operations for neurite detection, which can be challenging with overlapping neurites and high background fluorescence. However, it requires minimal manual input and does not depend on a large, well-annotated training dataset, which can be time-consuming to generate and may not always be feasible for certain applications. Thus, incorporating deep learning-based segmentation models for neurite detection could improve measurement accuracy while maintaining the accessibility of CellProfiler. Additionally, a hybrid approach could enhance the interpretation and classification of complex cellular phenotypes, making it more powerful for large-scale experiments and broadening the scope of its applications.

4.1.2. Neuromorphogenesis defects in LS as a target for drug discovery

Given the need to discover new mechanistic targets in the treatment of LS, I explored whether the observed neuromorphogenesis defects in *SURF1*-derived neurons could serve as a target in a phenotypic-based drug screen. To conduct this screen, I employed a robust, scalable model to differentiate neurons in a fast and reproducible manner, via the overexpression of *NGN2* transcription factor.

Following the original protocol, I employed a lentiviral delivery system for the constitutive expression of rtTA and tetracycline-inducible expression of *NGN2* driven by tetO promoter (Zhang et al., 2013). However, the classic *NGN2* protocol bypasses the neural progenitor state, which is critical in normal neurodevelopment. This limitation is particularly relevant for modeling LS, where NPCs are primarily affected (Inak et al., 2021; Lorenz et al., 2017). To overcome this drawback, I adapted the protocol and used NPCs as the starting point of the assay. Additionally, I excluded the use of astrocytes to see the intrinsic cellular factors impairing neuronal morphogenesis.

Using this adapted approach and the published CellProfiler pipeline (Lickfett et al., 2022), I identified a decrease in neuronal morphogenesis as early as five days after *NGN2*

induction, characterized by a significant decrease in the number of neurons and a tendency towards displaying shorter neurites (Menacho et al., in revision).

Neuronal morphogenesis is an energetically demanding process that requires proper mitochondrial functionality and its impairment affects neuronal development (Brunetti et al., 2021; Lisowski et al., 2018; Rangaraju et al., 2019; Schon & Przedborski, 2011; Trigo et al., 2022).

Accordingly, an alteration of neuronal morphogenesis has not only been shown in *SURF1*-deficient models (Inak et al., 2021; Menacho et al., in revision) but also in other patient-derived stem cell neuronal models of primary mitochondrial disease. MERRF (Myoclonic Epilepsy with Ragged Red Fibers)-derived neurons with mutated tRNA^{Lys} (m.8344A>G) gene exhibited a slower growth rate with reduced expression of synaptic proteins (Wu et al., 2023). Retinal ganglion cells carrying m.11888G>A mutation in *MT-ND4* gene displayed shorter neurite length with fewer connections (Wu et al., 2018). Shorter dendrites with fewer branches containing fewer synapses were also observed in iNs with high heteroplasmy levels for mtDNA mutation m.3243A>G (Klein Gunnewiek et al., 2020). In addition, organoids carrying the same m.3243A>G mutation exhibited severe alteration of motor neuron differentiation linked to hyperactive Notch signaling (Winanto et al., 2020), which has been shown to maintain progenitor states (Tan et al., 2016).

Collectively, these reports suggest that altered neuromorphogenesis is a shared neuropathological feature across different primary mitochondrial disorders, highlighting the importance of this phenotype for therapeutic intervention.

In this thesis, I furthermore aimed to identify drug compounds that would improve neuronal morphogenesis in patient-derived iNs carrying *SURF1* mutations. To assess their efficacy, I evaluated selected compounds in modulating early neuromorphogenesis after a single dose at three different drug concentrations. This setup prioritizes the identification of compounds that directly impact neuronal morphogenesis and may overlook those that could eventually correct the morphogenesis defects over time.

Consequently, this study serves as a hypothesis-driven drug discovery effort, leveraging human neurons to screen compounds previously identified in two independent approaches: (i) a deep-learning screen designed to retrieve compounds that enhance neuronal cell fate commitment based on single-cell RNA sequencing (scRNA-seq) data from *SURF1* and control cerebral organoids (Inak et al., 2021); and (ii) a SHY-knockout yeast screen used to identify repurposable compounds that increase viability (Menacho et al., in revision). The integration of these two screens with the iNs morphogenesis validation led to the identification of two main compounds: Talarozole and Sertaconazole. Both compounds increased neuronal number and neurite length following a single 10 μ M dose, while higher concentrations (50 μ M) were toxic.

Talarozole and Sertaconazole belong to the chemical class ofazole compounds, characterized by the presence of a ring structure containing a nitrogen atom and at least

one other non-carbon atom. Interestingly, multiple azole compounds were among the top candidates in the yeast viability screen. This class of compounds, widely used as antifungal agents within the medical field, have been shown to influence neuronal health, displaying both neurotoxic and neuroprotective effects (Giorgi et al., 2015; Hennegan et al., 2024; Jang et al., 2014; Sherman & Bang, 2018; Toodle et al., 2022). The specific outcome may depend on the dosage and duration of exposure, but more importantly on which azole compound is being tested.

To fully validate the beneficial effects of Talarozole and Sertaconazole in aiding neuronal morphogenesis in the context of LS, I proceeded to test the effects of long-term treatment with these azoles on 3D MOs (Menacho et al., in revision; discussed in section 4.2). This validation would provide a more physiologically relevant setting to determine whether the observed effects on neuronal morphogenesis in iNs extend to other populations and functions.

Although evaluating the effects of Talarozole and Sertaconazole on neuronal functionality was beyond the scope of this thesis, it would be valuable to determine whether these compounds enhance the electrophysiological properties of iNs. Patient-derived *SURF1* midbrain dopaminergic neurons showed electrophysiological defects consistent with their decreased maturation levels (Inak et al., 2021). Using multi-electrode arrays (MEA), patch-clamp or calcium imaging in combination with transcriptomic analysis would assess whether the observed structural changes could translate into functional recovery and shed some light onto the molecular changes leading to improvement.

In the context of mitochondrial diseases, this strategy was employed by Gunnewiek and colleagues to assess the effect of the novel antioxidant Sonlicromanol in restoring the electrophysiological dysfunction of m.32434A>G iNs (Klein Gunnewiek et al., 2021). Neurons with high heteroplasmy level for m.32434A>G display reduced OXPHOS with downregulation of synaptic markers and less mitochondria at presynaptic sites, leading to altered neuronal network activity (Klein Gunnewiek et al., 2020, 2021). In this article, Sonlicromanol partially reversed this phenotype, providing molecular and functional evidence for potential positive effects exerted on neuronal-specific function reported in a previous study (Janssen et al., 2019). Sonlicromanol is currently under clinical trial for the treatment of MELAS (Smeitink et al., 2025).

The phenotypic pipeline developed in this study could be broadly applicable to other mitochondrial diseases, as iPSC-derived neuronal models of conditions such as LS, MELAS, MERRF, and LHON commonly exhibit defects in neuronal morphogenesis and synaptic function (Inak et al., 2021; Klein Gunnewiek et al., 2020; Menacho et al., in revision.; Wu et al., 2023; Yang et al., 2020). In this context, targeting neuronal morphogenesis represents a novel therapeutic avenue for these disorders. Moreover, integrating this phenotypic approach with functional assays would strengthen the therapeutic outcome and enhance its utility in defining key molecular pathways to tackle mitochondrial diseases.

4.2. MIDBRAIN DOPAMINERGIC ORGANIDS FOR DISEASE MODELLING AND DRUG DISCOVERY

4.2.1. Generation and characterization of NPC-derived MOs

NPC-derived MOs from control iPSC lines were grown and characterized after 30 days of differentiation using immunostaining and scRNA-seq.

Immunostaining of day 30 MOs indicated the presence of a high proportion of neurons, shown by the expression of MAP2 and NeuN, co-existing with a population of neural precursor cells, identified by the expression of SOX2 and NESTIN (Menacho et al., in revision). Differentiation towards a dopaminergic fate was confirmed by assessing the expression of tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine synthesis, and the expression of NURR1, a transcription factor known to drive the dopaminergic fate (Sakurada et al., 1999; Zetterstrom et al., 1997).

Unlike the cortex, the midbrain does not display the typical layer organization observed in cortical structures which can be replicated when generating cerebral and cortical organoids (Lancaster et al., 2013; Pasca et al., 2015). Instead, NPC-derived MOs displayed a self-organized and homogeneous structure with neural progenitors primarily populating the core, while a dense layer of neurons formed in the periphery (Renner et al., 2020).

Analysis of scRNA-seq of MOs at day 35 of differentiation identified four populations based on their different degree of maturation, consisting of (i) proliferative progenitors, (ii) progenitors, (iii) maturing neurons, and (iv) mature neurons. Mature neurons comprised 40-60% of the total population (Lisowski et al., 2024). Benchmarking of our MOs to previously published NPC-derived MOs (Zagare et al., 2022) revealed that our organoids contained 30.9% midbrain dopaminergic neurons (mDANs), compared to day 35 and day 70 MOs containing 7.6% and 20.2% mDAN, respectively (from Zagare et al., 2022) (Menacho et al., in revision). Thus, given our primary focus on early neuronal pathology and dopaminergic vulnerability, we identified days 28-35 as suitable time points to investigate the disease phenotypes caused by our mutations of interest in MOs.

A clear limitation of using this early time point for analysis is the reduction in cellular complexity, as seen by the absence of astrocytes. Our scRNA-seq identified a population expressing glial markers (14.2% of cells), which clustered with progenitors, suggesting a radial glial-like transcriptomic profile rather than mature astrocytes (Kriegstein & Alvarez-Buylla, 2009). Gliogenesis occurs at later stages of development after neurogenesis occurs (Kriegstein & Alvarez-Buylla, 2009). Thus, longer culturing would be required to obtain astrocytes in MOs (Zagare et al., 2022; Monzel et al., 2017; Renner et al., 2020). While the presence of astrocytes would enhance the biological relevance of the model, the use of early time points of differentiation allowed the study of progenitor- and neuronal-specific

phenotypes and drug responses without adding additional complex inter-cellular interactions.

Despite reduced cellular complexity, MOs were shown to be functional at early stages of development (Renner et al., 2020). Calcium imaging using Fura-2 AM revealed spontaneous calcium signals in ~50% of cells, indicating viable and functional neurons (Menacho et al., in revision). However, the observed calcium signals lacked synchronicity, a hallmark of network activity. This is likely due to the early time-point analyzed and the consequent absence of astrocytes, which are essential for synapse formation and maturation, enhancing neuronal connectivity and network (Slezak & Pfrieder, 2003).

In summary, NPC-derived MOs offered a homogeneous viable cell population enriched with dopaminergic neurons in a short time. While lacking cellular complexity, this model provides a robust and cost-effective platform for studying early neuronal defects in the context of mitochondrial dysfunction in HD and LS.

4.2.2. NPC-derived MOs for the study of mHTT in neurodevelopment

HD is a neurodegenerative disorder affecting the striatum and substantia nigra, caused by CAG expansions in the HTT gene. Wild-type (WT) HTT is involved in neuronal differentiation, axonal growth, and synaptic plasticity (Buren et al., 2016; Reiner et al., 2003; Trushina et al., 2004; Usdin et al., 1999). To gain mechanistic insights into how mHTT impacts human development, we generated neurons and organoids from engineered isogenic iPSC lines where mHTT with 70Q length was introduced in both *HTT* alleles (Lisowski et al., 2024). In this work, I contributed by generating MOs from wild-type (WT/WT) and mutant (70Q/70Q) NPCs, performing immunostainings and preparing samples for scRNA-seq.

MOs with 70Q/70Q HTT exhibited reduced size and an increased proportion of mature neurons over progenitors and proliferative progenitors based on scRNA-seq analysis (Lisowski et al., 2024). The observed premature neuronal differentiation likely depleted the progenitor pool, impairing proliferation and impacting the overall size of these organoids. Similar effects of mHTT altering the proliferation of neural progenitors and accelerating neuronal commitment have been reported in mice and human fetuses (Barnat et al., 2020; Molina-Calavita et al., 2014). Additionally, a premature commitment and posterior imbalance of neuronal composition and function have also been observed in other disorders, such as Parkinson's disease (Zagare et al., 2022) and ASD (Paulsen et al., 2022; Pighi et al., 2023).

Given mitochondria's key role in regulating stem cell proliferation, maintenance, and differentiation (Khachik et al., 2019), the premature neuronal commitment observed in our HD brain organoid models may be linked to mitochondrial dysfunction. Further experiments on 70Q/70Q neural cells revealed a mitochondrial dysfunctional signature consisting of disrupted mitochondrial dynamics, cristae morphology, and bioenergetic dysfunction with alterations of RC IV of ETC (Lisowski et al., 2024). Specifically, 70Q/70Q

NPCs exhibited mitochondrial cristae oriented longitudinally to the OMM instead of transversally (Lisowski et al., 2024). This abnormal orientation of mitochondrial cristae could disrupt the optimal spatial organization of the ETC, leading to impaired OXPHOS and compromising the overall mitochondrial function. The alteration of mitochondrial cristae has been suggested to be caused by the presence of mHTT (Costa et al., 2010; Hering et al., 2017). Consequently, the ablation of the polyQ tail in 0Q/0Q NPCs led to the restoration of normal cristae direction (Lisowski et al., 2024).

Our findings show the existence of a neurodevelopmental defect preceding the presence of mHTT aggregates and neurodegeneration in our organoid models (Lisowski et al., 2024), supporting similar evidence of neurodevelopmental alterations in HD fetuses and iPSC-derived neural models (Barnat et al., 2020; Kaye et al., 2022; Reiner et al., 2003).

Identifying how mHTT alters the neurodevelopmental process might be key to developing new therapeutic avenues. Our transcriptomic analysis across different neurodevelopmental stages identified a consistently downregulated signature in mHTT models: the mitochondrial protein CHCHD2. Accordingly, immunostainings of 70Q/70Q MOs exhibited decreased expression of CHCHD2 protein when compared to WT/WT organoids (Lisowski et al., 2024). Overexpression of CHCHD2 ameliorated the mitochondrial morpho-dynamics defects observed in 70Q/70Q NPCs, suggesting that mHTT could lead to CHCHD2 dysregulation, which in turn impairs mitochondrial function and eventually affects neuronal commitment. Accordingly, future studies could explore the therapeutic potential of CHCHD2 modulation and its capacity to restore normal progenitor dynamics and neuronal commitment.

Despite these alterations, neurogenesis still occurred in the presence of mHTT, but the functionality of these neurons was not tested. Given that early synaptic and network dysfunctions have been reported in other HD models (Milnerwood & Raymond, 2010), it is plausible that neurons derived from 70Q/70Q NPCs may exhibit functional deficits. Similarly, premature commitment of progenitors has been shown to affect neuronal diversity and functionality in organoid models of ASD (Paulsen et al., 2022). Additionally, these alterations may increase these neurons' susceptibility to stress, ultimately making them vulnerable to degeneration.

In HD, the selective vulnerability of striatal and midbrain dopaminergic neurons to neurodegeneration has been the hallmark of the disease. Future investigations will determine whether mHTT disrupts dopaminergic differentiation in our MO models. Moreover, the interplay between mitochondrial dysfunction, CHCHD2 downregulation, and dopaminergic vulnerability could be further explored.

Finally, human MOs offer a valuable platform for screening potential therapeutic interventions. Subsequent studies could assess whether pharmacological strategies restoring mitochondrial morpho-functional alterations – such as rescuing CHCHD2 expression – can correct the faulty neuronal commitment observed in our work. Combined with functional assessments of dopaminergic neurons, this technology could provide

critical insights into early neuronal dysfunction and novel therapeutic targets to prevent or delay the onset of HD.

4.2.3. Modelling LS with MOs

In this project, I devised a workflow to compare MOs derived from LS patients to their isogenic controls, aiming to discover disease-relevant phenotypes to test potential treatments (Menacho et al., in revision). To accomplish this, I utilized whole-aggregate-based readouts, such as size and lactate release, which enabled long-term monitoring of live-cell MOs throughout their differentiation. Additionally, I performed endpoint analysis, including molecular, histological, and functional assessments, to investigate the neuronal populations.

Brain organoid size is commonly employed as a proxy to mimic mega- and microcephaly *in vitro* (Lancaster et al., 2013; Mariani et al., 2015), as these phenotypes are linked to progenitor proliferation levels within the organoid. Cerebral organoids carrying *SURF1* mutations exhibited an imbalance between proliferation and maturation (Inak et al., 2021). Consistently, LS MOs showed aberrant size and an increased growth rate. This increase was apparent during the first phase of the protocol, when NPCs remained proliferative, and persisted during the maturation phase, when precursor cells should commit to the neuronal fate and abandon the cell cycle.

Increased proliferation is often accompanied by higher metabolic expenditure linked to a glycolytic profile over mitochondrial OXPHOS. Patient-derived MOs produced and secreted higher lactate in the media compared to their isogenic controls (Menacho et al., in revision). This increased lactate production mirrors the elevated lactate concentration in blood and cerebrospinal fluid observed in patients with *SURF1* mutations (Sofou et al., 2014; Stenton et al., 2022; Wedatilake et al., 2013) and presents a potential biomarker for assessing pharmacological interventions early in the differentiation without disrupting the organoid integrity.

Beyond its role as a glycolytic byproduct, lactate functions as a signaling molecule regulating diverse cellular processes such as inflammation and synaptic function (Li et al., 2022; Wu et al., 2023). Increased lactate production has been linked to aberrant proliferation in cancer (Li et al., 2022) and to neurodegeneration in various diseases (Wu et al., 2023), but also exhibits neuroprotective effects against oxidative stress (Taufenberger et al., 2019). In our system, we hypothesized that the elevated lactate levels observed on day 9 suggest an underlying metabolic defect, consistent with the aberrant glycolytic profile previously reported in NPCs carrying *SURF1* mutations (Inak et al., 2021; Menacho et al., in revision).

Building on our hypothesis that *SURF1* mutations promote a hyperproliferative, glycolytic state in neural progenitors, we assessed neuromorphogenesis defects in LS MOs. Evaluation of neuronal morphogenesis via immunostaining of microtubule-associated

protein, MAP2, and the pan-axonal marker SMI312, revealed an aberrant neurite organization of both dendrites and axons, respectively, and a qualitative decrease in neurite number (Menacho et al., in revision). *SURF1* neurons failed to form proper connections across the entire organoid, instead clustering into regionally restricted networks. These results are in line with published reports of disorganized cytoarchitecture and dysregulated expression of neuronal markers in LS brain organoids (Inak et al., 2021; Romero-Morales et al., 2022).

The use of region-specific brain organoids enables the study of desired neuronal populations such as the midbrain dopaminergic neurons. LS MOs showed a significant reduction in TH intensity, indicating a decreased population of dopaminergic neurons.

However, solely basing the presence of dopaminergic neurons on TH expression at a single point impedes discerning whether this reduction stems from a failure to commit to the dopaminergic lineage, a lack of TH expression, impaired maturation, or the progressive loss of this neuronal subtype. This organelle is essential for maintaining calcium and reactive oxygen species homeostasis, regulating stemness, and controlling apoptosis (Picard & Shirihi, 2022). Given their involvement in these vital functions, it is not surprising that mitochondrial dysfunction is linked with a variety of human diseases (Suomalainen & Nunnari, 2024)

This is particularly evident for neurological disorders, where mitochondrial alterations have been reported in neurodevelopmental conditions such as ASD, Rett syndrome, and Fragile X syndrome (Khaliulin et al., 2025; Rojas-Charry et al., 2018; Valenti & Vacca, 2023). However, the calcium dynamics in LS MOs were significantly altered, displaying reduced time to peak and increased peak amplitude compared to controls. These differences in calcium events may result from developmental stage disparities between patient and control neural cells, or an intrinsic dysregulation of calcium homeostasis in the cell.

Further experimental analysis would be required to answer this question. Nevertheless, it is not farfetched to hypothesize a possible dysregulation in calcium homeostasis given the role of mitochondria in calcium buffering and the tight link between mitochondrial ATP production and calcium homeostasis (Rizzuto et al., 2012). In fact, *Surf1*^{KO} mice and *Drosophila* have been shown to display impaired mitochondrial calcium buffering (Da-Rè et al., 2014; Dell'Agnello et al., 2007). Moreover, human models of LS carrying mutations in complex I (*NDUFS4*, *MT-ND5*) and complex V (*MT-ATP6* gene) that displayed reduced ATP production and altered mitochondrial membrane potential also exhibited altered calcium homeostasis (Galera-Monge et al., 2020; Lorenz et al., 2017; Visch et al., 2006). These findings suggest that mitochondrial dysfunction in LS could contribute to the observed calcium signaling abnormalities.

Despite harboring the mutation since birth, the onset of symptoms in infants with LS typically occurs after an episode of metabolic decompensation – such as infection or malnutrition – that challenges the system beyond its capacity to recover. To mimic such

metabolic decompensation *in vitro*, we applied a 2-minute metabolic stressor that transiently inhibited glycolysis and OXPHOS, and then we assessed the response of control and patient-derived MO using calcium imaging. The metabolic stressor comprised glucose-free artificial cerebrospinal fluid (ACSF), the glycolytic inhibitor 2-deoxyD-glucose (2-DG), and the complex IV blocker sodium azide.

Metabolic stress induced a rapid increase in intracellular calcium concentration in both control- and patient-derived MOs, as previously reported in control cortical organoids (Petersilie et al., 2024). However, LS MOs exhibited reduced peak amplitude and a shorter decay time, suggesting an altered response to metabolic stress (Menacho et al., in revision). This differential response to metabolic stress could be due to a differential expression or function of calcium channels, baseline intracellular calcium levels, or other compensatory mechanisms. While our current setup does not resolve the underlying cause, we speculate that such differences may influence neuronal vulnerability or resilience to metabolic insults. For instance, *Surf1*^{KO} mice exhibited increased resistance to glutamate-induced, calcium-mediated excitotoxicity, suggesting that altered calcium homeostasis may confer differential susceptibility to stress (Dell'Agnello et al., 2007).

Altogether, our findings highlight key metabolic and functional abnormalities in LS MOs carrying *SURF1* mutations. One caveat of this study is the lack of targeted functionality assays, such as addressing network functionality via MEA assay or assessing dopamine release, which requires a higher degree of maturation that could only be reached with longer culturing times. Additionally, the analysis of the cellular composition of patient-derived MOs with scRNA-seq could shed some light on the observed differences in dopaminergic neurons and help identify key dysregulated pathways in this model. While future studies could delve deeper into functionality assays, the findings presented in this work pave the way for drug validation in patient-derived organoids, exhibiting a high potential therapeutic value to improve the outcome of drug discovery processes.

4.3. DRUG DISCOVERY PIPELINES IN LS IDENTIFY AZOLE COMPOUNDS

In section 4.1, I described the development of a HCI analysis platform to measure neuronal morphogenesis in NPC-derived iNs and its application in validating hit compounds identified in two independent screening approaches: an *in silico* drug screening and an *in vitro* screening in yeast.

The first screening utilized a deep-learning neural network trained to identify compounds that enhance neuronal cell fate commitment. This model identified transcription factors that were differentially expressed in the scRNA-seq of *SURF1* and isogenic control cerebral organoids (from Inak et al., 2021). This algorithm predicted a total of 27 compounds targeting pathways involved in cell fate differentiation, either from radial glia to neuronal intermediate progenitors (strategy 1) or directly from radial glia to neuron (strategy 2). From this list, I tested nine compounds at three different concentrations and identified *Talarozole* as the most effective compound, promoting neurogenesis and increasing neurite length of LS iNs (Menacho et al., in revision).

The second screening leveraged a mutant strain for the *SURF1* homologue in yeast that was systematically tested against a library of 2,500 compounds in a viability assay. Among the top 50 hit compounds, many belonged to the azole family. From this subset, I selected and tested four compounds in Leigh iNs, and identified *Sertaconazole* as the most effective in enhancing neuronal morphogenesis (Menacho et al., in revision).

Following the identification of *Talarozole* and *Sertaconazole* in the 2D iN model, I examined their long-term effects in 3D MOs to assess whether their impact on neuronal morphogenesis extends to more physiologically relevant models. First, I determined the maximum tolerated dose that did not cause significant cell loss, as assessed by a viability assay. This resulted in 0.1 μ M *Sertaconazole* and 1 μ M *Talarozole* being selected for further experiments (Menacho et al., in revision). These concentrations were used to evaluate whether four weeks of treatment could rescue the organoid size reduction, elevated lactate release, defective connectivity and TH expression observed in LS MOs (Menacho et al., in revision).

Four-week treatment with *Talarozole* and *Sertaconazole* resulted in a reduction of size and lactate release in LS MOs, although the defects were not fully restored to control levels (Menacho et al., in revision). Treatment qualitatively improved neuromorphogenesis defects, with increased dendritic and axonal connections observed across the organoid structure (Menacho et al., in revision). Additionally, *Talarozole* significantly increased TH expression, as measured by immunostaining intensity, suggesting an effect on dopaminergic neuron differentiation or survival (Menacho et al., in revision). These findings

suggest that Talarozole and Sertaconazole improve neuronal morphology and some metabolic parameters in LS MOs, albeit without fully correcting the underlying mitochondrial dysfunction.

This partial rescue implies that both compounds may modulate downstream cellular pathways, and several key questions remain to be addressed. For instance, the observed reduction in organoid size and lactate release could indicate the correction of the aberrant hyperproliferative profile that LS NPCs exhibited in previous reports (Inak et al., 2021). To follow up on this, proliferation assays could clarify whether the treatment affects cell cycle dynamics.

In addition, further metabolic analysis could assess whether long-term treatment with these compounds promotes any metabolic rewiring in these organoids. For instance, performing a metabolic profile with the Seahorse 3D assay could shed some light on the mechanisms by which long-term treatment with Talarozole and Sertaconazole leads to decreased lactate release. Given that cell fate commitment requires a metabolic shift that is impaired in mitochondrial diseases (Brunetti et al., 2021), it would be interesting to understand whether pushing fate commitment would elicit any metabolic rewiring.

The collective results suggest that long-term treatment with Talarozole and Sertaconazole increases neuronal cell fate commitment and the presence of dopaminergic neurons (after treatment with Talarozole), albeit additional experiments are needed to corroborate these findings. In this regard, scRNA-seq could help determine whether treatments alter the population distribution between NPCs and neurons, and whether there is an increase in the percentage of dopaminergic neurons based on the expression of a subset of markers. This is crucial because increased TH intensity may not necessarily correspond to an increase in the number of dopaminergic neurons. In fact, the observed increase in TH could stem from enhanced neurogenesis or reduced neuronal apoptosis. Assessing markers of cell death and neuronal survival would further elucidate these possibilities.

Our assessment of neuromorphogenesis changes after treatment is limited by the lack of imaging parameters that could fully capture the differences between patient and control organoids in connectivity and neurite content.

Future improvements on image acquisition and analysis with machine learning programs may be able to better capture the differences in neurite distribution and content. For instance, the use of free algorithms for unsupervised image analysis, such as CellProfiler Analyst, could help identify and classify unbiased patterns between “healthy”, “disease” and “treated” conditions without the need of predefining specific differential features such as signal intensity. One limitation for this kind of analysis is the need of having a training dataset so that the algorithm can rightfully categorize each image, for which a high number of organoids would be needed. To circumvent this and increase the throughput of this readout, it would be best to use automated systems for image acquisition, such is the Operetta HCI analysis system (Revvity), which has been previously used for compound screening in MOs (Renner et al., 2021).

Finally, future experiments using longer culture conditions could assess neuronal connectivity defects via calcium imaging or MEA assay and determine specific functional readouts such as defects in dopamine release. Together, these functional analyses could address whether the beneficial effects of long-term treatment with Talarozole and Sertaconazole would eventually lead to improvements in neuronal maturation and proper synaptic activity.

Despite presenting some limitations, this work identified two repurposable compounds for the treatment of LS phenotypes in MOs: Talarozole and Sertaconazole. An important consideration when evaluating the effects of the drugs and their translational potential is the timing of the treatment selected. In our model, Talarozole and Sertaconazole were administered during neurogenesis, enhancing neuronal fate commitment. However, in LS patients, the predominant phenotype observed is progressive neurodegeneration rather than impaired neurogenesis, with symptoms typically emerging in early childhood after an initial period of normal development (Rahman, 2023). Despite this, growing evidence from human iPSC-derived neural models of various mitochondrial disorders suggests defective neuronal development and maturation that could predispose neurons to greater vulnerability and ultimately lead to neurodegeneration (Brunetti et al., 2021; Heiduschka & Prigione, 2025; Henke et al., 2024).

Advances in early diagnosis are now opening new windows for intervention during neurodevelopment, which could provide long-term neuroprotective benefits (Adelizzi et al., 2024; Bottani et al., 2020). Moreover, neurogenesis continues after birth and into adulthood, raising the possibility that these treatments could still be effective at later time points by promoting neuronal maturation and synaptic function—an aspect that warrants further investigation. Given that LS is characterized by both impaired neurodevelopment and neurodegeneration, a combination of treatments enhancing neuronal differentiation with those aimed at supporting neuronal survival could offer a more comprehensive therapeutic strategy. Defining the optimal treatment and the best time window will be critical for potential clinical applications.

While iPSC-derived models offer valuable mechanistic insights, additional preclinical validation is necessary to determine whether enhancing neurogenesis could have a protective effect in vivo. In this regard, animal models such as the *SURF1*^{KO} piglet, which exhibits early mortality and abnormal brain cytoarchitecture (Quadalti et al., 2018), could provide critical insights into whether these compounds improve functional outcomes in a more complex system.

Future research should aim to integrate findings from in vitro and in vivo models to refine treatment strategies, ultimately determining whether targeting neurogenesis can provide long-term neuroprotection. Combining pharmacological approaches with metabolic interventions may further enhance therapeutic outcomes, offering new avenues for addressing LS and other neurometabolic disorders.

4.4. TALARAZOLE AND SERTACONAZOLE EXERT PLEIOTROPIC EFFECTS IN LS NEURAL MODELS

4.4.1. CYP26 as common molecular target for azole compounds

In this thesis, I devised a workflow to validate compounds aiding neuromorphogenesis in patient-derived 2D neurons using a HCI pipeline (discussed in section 4.1). Through this approach, I identified two compounds that increased neurogenesis in Leigh iNS: Talarozole and Sertaconazole. Further investigation in MOs carrying *SURF1* mutations validated their ability to ameliorate the disease phenotype observed in these models (discussed in section 4.3). This partial rescue suggests that while these compounds may not correct the primary mitochondrial dysfunction, they modulate downstream cellular pathways. Thus, our phenotypic-based validation highlights new drug repurposing strategies, though further investigation into their mechanisms of action is crucial.

Talarozole was identified through deep learning screening aimed at finding compounds that enhance cell fate commitment. This compound acts as an inhibitor of the cytochrome P450 enzyme CYP26 isoenzymes (CYP26A1 and CYP26B1), which degrade retinoic acid (RA), thereby elevating endogenous RA levels and enhancing its biological effects (Pavez Lori  et al., 2009; Sakamuru et al., 2024).

Sertaconazole, an antifungal agent, was discovered via viability screening using a yeast model with mutations in the *SURF1*-homologue gene *SHY*. Sertaconazole selectively inhibits the fungal cytochrome P450 enzyme 14 -demethylase, disrupting ergosterol synthesis and compromising fungal cell wall integrity (Carrillo-Mu oz et al., 2013). While its selectivity for fungal CYP51 is much higher than for the human enzyme, Sertaconazole may still be able to impact cholesterol biosynthesis by inhibiting human CYP51, albeit with lesser effectiveness (B langer et al., 2011).

Additionally, many azole antifungals (e.g., ketoconazole, fluconazole) are known to inhibit multiple CYP enzymes, including those involved in retinoid metabolism (Sakamuru et al., 2024). Therefore, although Talarozole and Sertaconazole theoretically act through distinct pathways, they share structural similarities and target cytochrome P450 enzymes.

Given these parallels, I investigated their cellular effects in tandem. In collaboration with Dr. Andrea Zaliani (Fraunhofer Hamburg), we compared the structural similarity of Talarozole and Sertaconazole and predicted their binding potential to the iron center of CYP26 enzymes. Using Talarozole's conformation as template, this *in silico* modeling suggested that Sertaconazole could adopt a 3D conformation that could allow its interaction with the heme catalytic center of CYP26 with a score similar to the one obtained with Talarozole (Menacho et al., in revision). Consistently, treatment with both compounds

altered *CYP26* gene expression in iNs, indicating a possible shared target (Menacho et al., in revision).

Our bulk transcriptomic analysis of midbrain dopaminergic neurons (Inak et al., 2021) revealed elevated *CYP26A1* and *CYP26B1* expression, along with the downregulation of RA receptors (*RARA*, *RARB*, *RORA*, *RORB*) in *SURF1* models (Menacho et al., in revision), indicating the dysregulation of the RA pathway.

RA plays pleiotropic roles in neurodevelopment and is particularly important for the generation of dopaminergic neurons (Maden, 2007). Thus, the transcriptional dysregulation of the RA pathway is consistent with defective neuromorphogenesis and decreased TH⁺ neurons observed in our models. Accordingly, the inhibition of CYP26 with Talarozole should increase intracellular RA levels (Stevenson et al., 2017) and enhance midbrain specification, as evidenced by increased TH⁺ expression in our long-term treated MOs (Menacho et al., in revision).

These results support the involvement of the RA pathway in the pathomechanisms of our LS models, but additional experiments directly targeting this pathway are needed to confirm this mechanism. For instance, CRISPR-mediated knockout of CYP26 or partial silencing of RNA transcription of CYP26 could shed some light on the direct contribution of this pathway in our models. Additionally, the direct modulation of RA receptors using specific agonists could better dissect the molecular mechanism by which this pathway contributes to the observed phenotype.

Meanwhile, Sertaconazole did not correct this phenotype, suggesting a distinct mechanism of action, likely through the modulation of cholesterol synthesis via inhibition of CYP51; or a lesser effect on the RA pathway.

4.4.2. Azole compounds could not rescue impairments in mitochondrial transport and docking

Beyond regulating neurogenesis through transcription, RA signaling has been described to modulate neurite outgrowth by coordinating mitochondrial trafficking in the cell (Trigo et al., 2019). Defective neuronal morphogenesis is a common feature of mitochondrial disorders (Klein Gunnewiek et al., 2020; Wu et al., 2018; Wu et al., 2023), but the direct link with altered mitochondrial transport has not always been explored in these models.

We analyzed mitochondria trafficking in *SURF1*-mutant midbrain dopaminergic neurons and described higher proportion of moving mitochondria compared to stationary mitochondria both at 4 and 8 weeks of differentiation, suggesting impaired mitochondrial docking (Menacho et al., in revision). Since stationary mitochondria increase over time to support neurite branching and neuronal maturation, this finding supports previous observations of developmental defects in *SURF1*-mutant neurons (Inak et al., 2021).

Additionally, *SURF1*-mutant neurons and cerebral organoids displayed altered expression of key genes regulating mitochondrial transport (*KIF1A*, *KIF5A*, *KIF5C*) and docking (*SNPH*,

SYBU, TRAK2) (Menacho et al., in revision). These results are consistent with affected axonal mitochondrial transport in other mitochondrial disorders where neuronal morphogenesis is affected (Yang et al., 2020). Proper axonal mitochondrial transport is essential for synaptic function (Devine & Kittler, 2018), and up to 50% of synapses contain mitochondria at their presynaptic terminals (Rangaraju et al., 2014). Thus, it would be interesting to explore whether defects in mitochondria trafficking lead to depleted mitochondria content in synapses and an overall effect on synaptic puncta, as seen in other iPSC-derived neurons with defective neuromorphogenesis due to mitochondrial mutations (Klein Gunnewiek et al., 2020).

In our LS models, Talarozole and Sertaconazole promoted neuronal morphogenesis acutely in iNs and after long-term treatment in MOs. However, due to the technical challenge of performing *in vivo* imaging of neuronal mitochondria in 3D structures, we were not able to assess the effect of these two compounds on mitochondrial transport and anchoring with proper cellular resolution. Instead, we assessed the protein levels of SNPH and KIF5A, two of the top dysregulated genes in our transcriptomic dataset whose levels remained unchanged upon treatment with azoles (Menacho et al., in revision).

These results are insufficient to draw strong conclusions since changes in mitochondrial trafficking may not translate into a correction in the expression of these specific proteins. In fact, the effect of RA signaling on mitochondrial motility was exerted *via* HIF1- α signaling and mitochondrial anchoring was regulated by the chaperone GRP75. Thus, the next steps could explore the effect of azole treatments in reversing the proportion of moving mitochondria *versus* stationary mitochondria in midbrain dopaminergic neurons using appropriate *in vivo* imaging techniques. Additionally, it would be most interesting to dissect whether these effects may be mediated by RA signaling and explore the underlying mechanisms in more detail.

4.4.3. Talarozole and Sertaconazole distinctly impact NPC metabolism

RA exerts its cellular effects via two key pathways: the canonical retinoic acid receptor (RAR)-mediated pathway, which regulates genes essential for neurogenesis and differentiation through Wnt and SHH signaling (Janesick et al., 2015); and a non-canonical RXR/PPAR-dependent mechanism involved in lipid metabolism, mitochondrial biogenesis, and energy homeostasis (Noy, 2016). This distinction is crucial in understanding how these compounds could exert their beneficial effects in ameliorating the LS phenotype.

Given that the non-canonical RXR/PPAR-dependent RA pathway regulates energy homeostasis and that neuronal commitment requires a shift from glycolysis to OXPHOS, I next investigated whether Talarozole and Sertaconazole could facilitate this metabolic rewiring in NPCs using targeted metabolomics.

LS NPCs displayed high levels of glycolysis and lactate production that were not corrected by either of the treatments (Menacho et al., in revision). Within the TCA cycle, patient-derived NPCs exhibited elevated levels of succinyl-CoA and oxaloacetate. While Talarozole

partially rescued both defects, Sertaconazole mainly affected succinyl-CoA (Menacho et al., in revision).

Additionally, Talarozole also increased the AMP/ATP ratio, which was downregulated in patient NPCs (Menacho et al., in revision). Elevated AMP/ATP levels activate AMPK, an enzyme that acts as an energy sensor and promotes mitochondrial biogenesis through activation of the master regulator PGC-1 α (Cantó & Auwerx, 2010). Overexpression of PGC-1 α and pharmacological induction of AMPK activation led to increased mitochondrial biogenesis with partial recovery of COX activity in *Surf1*^{KO} mice (Viscomi et al., 2011). Given the therapeutic potential of mitochondrial biogenesis as a treatment for mitochondrial diseases (Bottani et al., 2020), future experiments should assess whether Talarozole could induce the generation of new mitochondria through the activation of AMPK pathway.

Besides the potential effects through RA pathway, azole compounds may inhibit the human CYP51A1, involved in the early steps of cholesterol synthesis (Trösken et al., 2006). To assess potential changes in lipid content, NPCs were treated acutely with Sertaconazole and Talarozole and stained against Bodipy and PFO (Perfringolysin O) to label neutral lipids and membrane-bound cholesterol, respectively (Menacho et al., in revision).

Bodipy staining revealed that Sertaconazole, but not Talarozole, increased neutral lipid content in all NPCs (Menacho et al., in revision). Inhibition of cholesterol biosynthesis by CYP51A1 inhibition may cause sterol intermediates to accumulate and be redirected toward neutral lipid storage (Olzmann & Carvalho, 2019). In parallel, PFO staining showed that Leigh NPCs had reduced membrane-bound cholesterol, partially rescued by both compounds, with Sertaconazole having a stronger effect (Menacho et al., in revision).

While we did not measure total cholesterol levels, our results showed that treatment with azole compounds affects cholesterol distribution in the cell. Aberrant cholesterol metabolism has been linked with defects in mitochondrial transmembrane proteins such as ATAD3A (Muñoz-Oreja et al., 2024), SLC25A46 (Janer et al., 2016) or SERAC1 (Wortmann et al., 2012) leading to Leigh (and Leigh-like) syndrome. Alteration of these transmembrane proteins leads to altered phospholipid composition in the mitochondrial membrane, ultimately affecting mitochondrial functions.

Membrane-bound cholesterol was significantly decreased in *SURF1* NPCs compared to their isogenic control. It would be interesting to assess whether this alters the fluidity and overall phospholipid composition of the mitochondrial membrane, and whether these change upon treatment with Sertaconazole and Talarozole. Additionally, given that these treatments may influence the overall lipid homeostasis within the cell, performing untargeted lipidomics could provide a full overview of the differential drug effect on lipid metabolism. In summary, Talarozole and Sertaconazole ameliorate the phenotypes observed in LS MOs, but they seem to act through distinct molecular pathways that may ultimately converge on similar cellular functions (Figure 10).

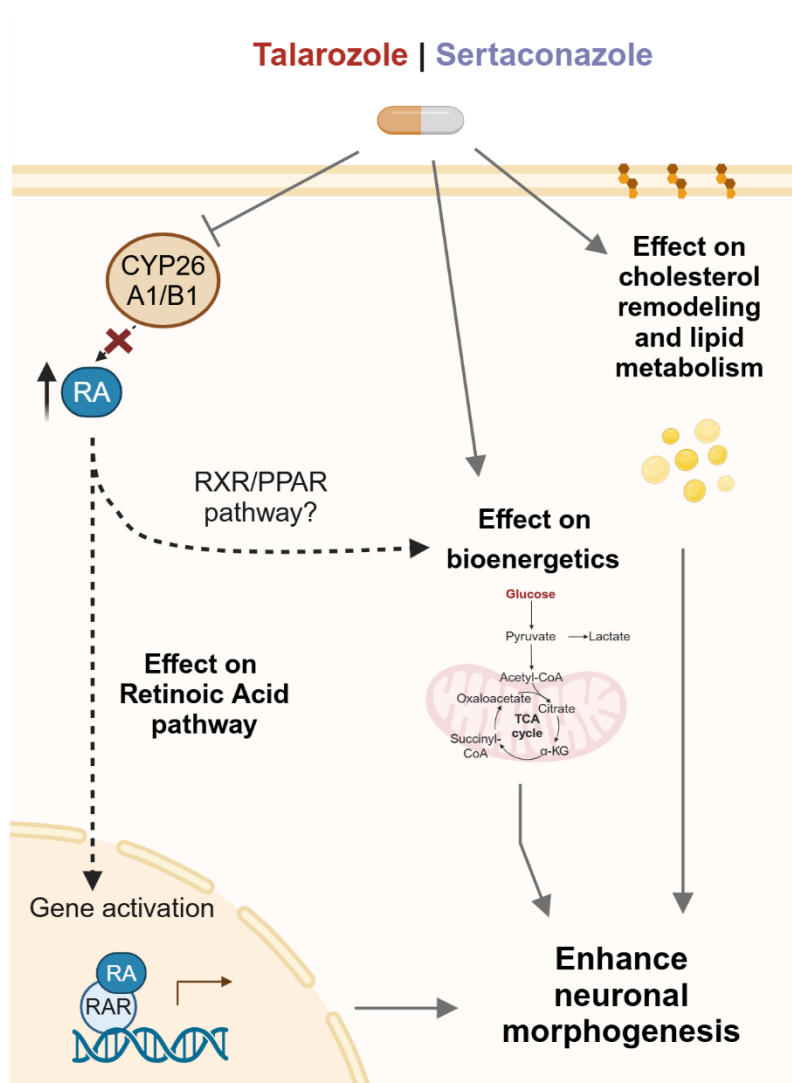


Figure 10 Talarozole and Sertaconazole pleiotropic molecular pathways converge on common cellular effect. Talarozole and Sertaconazole may act through the Retinoic acid (RA) pathway with different degrees of efficiency, potentially leading to differential effects in gene activation and other cellular effects modulated by RA. Among these, mitochondrial trafficking and anchoring seem to be unaltered by azole treatment, while both azoles differentially affect mitochondrial bioenergetics without correcting the underlying mitochondrial defect. These metabolic effects may be mediated by the involvement of non-canonical RA pathways, such as RXR/PPAR pathway, or the effect of azole compounds on cholesterol remodeling, potentially through the inhibition of the human CYP51A1 enzyme. Image created with Biorender.

Both compounds exerted positive effects, modulating organoid size, lactate production, and neuromorphogenesis. On the one hand, Talarozole distinctly increased the presence of dopaminergic neurons in the organoid, an effect most likely mediated by the canonical action of RA. On the other hand, Sertaconazole exerted a stronger effect on cholesterol homeostasis, likely through the inhibition of CYP51A1. However, due to their structural similarity, these azole compounds may share common molecular targets with distinct affinities, giving rise to pleiotropic effects in the cell.

A major limitation of this study is the lack of specific pathway manipulation to discern the specific contribution of each pathway to the amelioration of LS phenotype in our models.

Next steps should address this limitation with targeted genetic interventions to dissect the contribution of each pathway, validate their roles, and refine their therapeutic potential.

For instance, genetic knockdowns of *CYP26A1*, *CYP26B1*, or *CYP51A1* in patient-derived neurons could clarify their individual contributions to improving neuromorphogenesis defects in *SURF1* iNs. Additionally, integrating single-cell transcriptomics and lipidomics could provide a comprehensive view of the cellular adaptations induced by Talarozole and Sertaconazole, thereby elucidating their precise mechanisms of action.

Finally, the potential role of PPAR/RXR activation in promoting mitochondrial biogenesis remains unexplored in this study, despite its emerging relevance as a therapeutic strategy for LS. Notably, various pharmacological approaches targeting PPAR pathways, including Bezafibrate, thiazolidinediones, and more recently, cannabidiol, have shown beneficial effects in preclinical models of LS (Inak et al., 2021; Puighermanal et al., 2024). Notably, this pathway has been shown to be dysregulated in other neurodegenerative diseases and how promoting RXR pathway activation using Bexarotene was beneficial in preclinical models of Alzheimer's disease (Cramer et al., 2012) and HD (Dickey et al., 2016, 2017)

These treatments, like Sertaconazole and Talarozole, exert pleiotropic effects at the cellular level. While Sertaconazole and Talarozole converge to enhance neuronal fate determination, the precise contribution of distinct pathways to this outcome remains unclear. Future studies should investigate the interplay between the different mechanisms to offer new insights into disease-modifying therapeutic strategies.

5. CONCLUSIONS AND FUTURE PERSPECTIVES



Disease modeling encompasses the creation of a representative system that mimics the behavior of the disease to gain insight into the molecular mechanism of the pathology and evaluate the effectiveness of potential treatments. In this thesis, I employed iPSC-derived neural models, including NPCs, neurons, and regionalized brain organoids, to investigate how mitochondrial defects caused by mHTT and *SURF1* mutations can affect neuronal morphogenesis. By leveraging these models, I sought to address critical gaps in understanding neurodevelopmental impairments linked to mitochondrial defects and to establish new therapeutic avenues.

A key aspect of this work was the adoption and optimization of a protocol for generating MOs from NPCs. This protocol was chosen to overcome some of the main barriers limiting the widespread application of organoid models in drug discovery, such as reproducibility, scalability, and cost. Although MOS have been previously utilized for toxicity studies, their potential for modeling mitochondrial dysfunction in neurodegenerative diseases had not been fully explored. By establishing a systematic approach to assessing neurometabolic defects, I demonstrated that these models effectively recapitulate key neuronal features relevant to disease pathophysiology.

Our findings suggest that mHTT, causative of HD, induces mitochondrial morpho-functional defects leading to a decreased progenitor pool and increased early commitment towards neuronal fate (Lisowski et al., 2024) in MOs. Instead, *SURF1* mutations disrupt the metabolic rewiring required for proper neuronal differentiation, leading to impaired neuromorphogenesis, altered dopaminergic neuron integrity, and defective calcium activity in MOs. These results provide critical evidence that mitochondrial dysfunction is not merely a secondary consequence of neurodegeneration but plays a fundamental role in shaping early neuronal development.

Despite their value, MOS have inherent limitations, particularly in their cellular complexity at early differentiation stages. Future studies should extend differentiation timelines to explore long-term effects of mitochondrial dysfunction, particularly in relation to glial maturation, neuronal connectivity, and network synchronization. Additionally, assembling MOs with other regionalized structures, such as striatal and cortical organoids, could enhance our ability to model the defective circuitry and selective vulnerability of the basal ganglia and midbrain system in LS and HD.

In addition to elucidating disease mechanisms, this thesis establishes a foundation for pharmacological intervention by targeting defective neuronal morphogenesis in LS-derived neurons. By developing an HCI workflow to quantify neuronal morphogenesis defects, I identified hit compounds whose beneficial effects in ameliorating disease phenotypes I validated in MOS.

Through phenotype-based validation assays, I identified Talarozole and Sertaconazole as repurposable drug candidates for treating LS. The repurposing of Talarozole and Sertaconazole for LS presents both intriguing opportunities and clear challenges.

One clear challenge is their pharmacokinetic profile: designed for topical application, these drugs typically exhibit limited systemic absorption and may not readily cross the blood-brain barrier (BBB). Future *in vivo* studies are essential to determine whether they can be delivered systemically or whether alternative delivery methods such as intranasal administration or nanocarrier-based formulations, are required to increase their translational potential before treating patients suffering from LS.

Safety and tolerability also warrant careful evaluation. Like other azole-based drugs, these compounds may elicit off-target effects or toxicity at high doses. Dissecting the specific cellular pathways influenced by these molecules—including the RA, PPAR, and cholesterol pathways—could enable the identification of structurally or mechanistically related compounds with improved safety profiles. Notably, modulation of these pathways has yielded therapeutic benefits in preclinical models of other neurodegenerative diseases (Cramer et al., 2012; Dickey et al., 2017), underscoring their relevance in the context of LS.

While additional research is needed to fully elucidate the mechanisms of action of Talarozole and Sertaconazole, the use of iPSC-based neural models provides a powerful platform for uncovering disease-modifying pathways in LS and related disorders. This work paves the way for broader application of neuromorphogenesis-based readout in high-throughput compound screening, investigation of azole derivatives, and exploration of mitochondrial contributions to early neurodevelopment.

In conclusion, this thesis demonstrates the pivotal role of mitochondrial function in shaping neuronal development and positions defective neuromorphogenesis as a tractable target for therapeutic intervention. By integrating advanced iPSC-derived models with HCI-based phenotyping, this research offers valuable insights into disease pathogenesis and lays the foundation for future translational efforts in mitochondrial medicine and neurodevelopmental disorder research

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I sincerely thank my colleagues at Stem Cell Metabolism lab for their teamwork and help in the lab. Special gratitude goes to Annika, Selene, and Stephanie. Annika, you have always been there to listen to any problems since day 1, always had time for a much-needed coffee break, and have been a good friend, besides being a good colleague. Selene, thank you for all the brainstorming, the constructive feedback, and complaining sessions that helped me push the project forward. And Stephanie, thank you for always having a fresh podcast for our 8 a.m. sessions, for the coffee walks, for all the sarcasm and good mood in the long days, for being the social one, and for having the patience to deal with me in every state of my personality. You have been one of the cornerstones of my support system in Düsseldorf and I would have gone mad without your daily support. I can't wait to see you suffering in the humid heat of Barcelona.

I would like to thank our lab neighbors in the corridor of the Stoffwechsellabor for all their support, collaboration, help, and daily camaraderie. To Martina, Diran, Annette, and Tharsini at the very beginning, who gave me a warm welcome and helped me until the end. To Kim, Hubert and Wilfred, who always had time for a little visit, a little chit-chat in the corridor, ALWAYS with a beautiful, shining smile that made the rainy seasons more bearable.

Over the past five years, our labs have had to endure many challenges, starting with the COVID pandemic. We all met with face masks and social distancing, and bonded over difficulties that make a person want to quit. A lab on fire. The dehumidifiers. Lack of reagents, failing equipment. It has been a journey, and many people have come and gone, shaping the trip and leaving a mark, so thank you all for contributing in one way or another.

In this sense, some people start as secondary characters and stay in life as protagonists. I cannot go further without thanking the other cornerstone of my life in Düsseldorf. Giulia, I cannot find the words to thank you for becoming such a dear friend of mine and supporting me during my lowest times in this city. I already miss all the free therapy sessions and movie nights with you, roomie, so don't take too long to visit me.

This thesis has been a huge chapter in my life as an expat living in Germany for 7 years.

I started this long journey in 2017, when I moved to Heidelberg, and met my very first friend in this country. Angelika, I was lucky enough to start at the same time and sat together with you. Thank you for making the start of this journey so easy, and for becoming a dear friend of mine in such a record time. What a pity that fate did not bring me to Munich, I bet we would have had LOTS of fun! :)

Those very-long days would not have been the same without the support of my BioMed X family: Miguel, Eliana, Óscar, Vero, Nico. Thank you for the great times and for becoming my little international family for a year. This was an intense experience but I was lucky enough to share it with great people who helped me develop both personally and professionally. Dolores, I cannot thank you enough for all the advice and constant support. Thank you for adopting me as your little "kiddo" and guiding me through those tough times. I learned a lot from you.

And, of course, Heidelberg and Düsseldorf would not have been the same without the support of Annika in a pivotal time of this chapter in life. Sharing life amidst the isolation of the pandemic was a gift that brought great moments that I deeply appreciate. Though our paths eventually diverged, I remain grateful for all your support and presence during that time.

Nadie se hubiera imaginado que iba estar 7 años en Alemania, y muchos, incluida yo, no sabemos cómo lo he hecho. Lo que sí sé, es que no me hubiese ido al extranjero de nuevo si mi Erasmus en Trieste no hubiese sido una etapa tan importante en ese capítulo de mi vida. María, Bea, Elena, Omar, Miriam, Ali, mil años después os vuelvo a dar las gracias por ese gran año y esa familia tan bonita que formamos y que aún perdura.

Aunque si de verdad buscamos a los culpables de mi afán por hacer el doctorado, debo remontarme al principio de los tiempos y volver a agradecer una vez más al Astrolab. Abel, Arantxa y Raquel, muchísimas gracias por todas vuestras enseñanzas y todo el tiempo que invertisteis en esta mindu. Gracias por enseñarme tanto y por acogerme como una más entre vosotros, creasteis las bases de mi "yo" investigadora con mucho cariño y con cada año que pasa y más laboratorios que veo lo valoro más. Siempre me faltarán las palabras de agradecimiento por vuestro trabajo con este pollito, pero si no hubiese tenido esa experiencia tan bonita no me hubiese atrevido a hacer todo este recorrido, así que culpable sois.

7 años en Alemania. Siete. El tiempo vuela cuando miras atrás, pero pasa extremadamente lento cuando vives tiempos difíciles. Siete. Siete, que no hubiese podido aguantar sin el apoyo de mi familia y amigos, que me anclan y me crean un hogar, me vaya donde me vaya, cuanto me vaya.

A mis chicas del WIII ;). Esos primeros años de tortura universitaria han sentado las bases de este grupito de locas que sigue cumpliendo años. Gracias por todos esos viajes de desconexión total que me recargaban las pilas antes de volver, y por el apoyo inagotable a larga distancia. Bea, mi compi alemana, me ha gustado verte en distintas ciudades y etapas de este país, empezando por la entrevista de trabajo que me trajo aquí. No sé por qué hicimos esto, pero lo

hemos hecho y hemos sobrevivido, que es lo importante. Ahora sí, no vuelvo a seguir tus pasos... A mi grupo 2, Aida, Ana y Elena, porque nos lo pasábamos TAAAAAN bien en aquellas prácticas TAAAN horrorosas, que siempre que se pueda hay que agradecer la buenísima compañía. Aidi, gracias por dar siempre el punto de vista más razonable y sacarnos de nuestra esclavitud científica, y por salpimentar nuestros viajes con reggaetón. Seguiré tirándote los trastos, guapita. A mi gran compañera y desastrillo de laboratorio, Ana, gracias por tener siempre palabras cálidas de apoyo y comprensión. Elena, gracias por todas las llamadas de apoyo y motivación, por estar ahí como buenamente se ha podido, y por instarme incansablemente a ocupar espacio. Grupo 3, no os pongáis celosas, el apoyo incombustible del “nuevo” sector Sabadell ha sido clave en el desarrollo de esta tesis. A Maria, gracias por toda la validación de este saquito de estrés y emociones que somos, y por esos grandes abrazos de oso que me dabas en cada visita. Y a Lorena, porque aunque me riñas te quiero mucho, corasón. Espero seguir celebrando muchos más cumpleaños con vosotras en el Delta. Y más bodas. Y bautizos. De mí ya no os libráis je, je.

Obviamente me dejo a una persona del Wii, pero es que mi alma gemela del Matri es omnipresente en mi vida. Nat, te podría haber puesto en los agradecimientos junto a mi familia, ya lo sabes. Es imposible agradecer tantos años de apoyo constante, de fidelidad y de darme la seguridad de que SIEMPRE vas a estar ahí. De que siempre VAIS a estar ahí, porque mi alma gemela real todos sabemos que es la tercera pata de esta trijea. Manu, Naty, gracias por acompañarme todos estos años, desde el principio de los tiempos, y no dejar que decaiga.

Pipe, Manu, Natalia, sois mi pequeña familia y os quiero mucho aunque tenga que explicar siempre qué significa este tatuaje y quede como una borracha. Gracias por todo, todito, todo.

Aunque para apoyo incondicional, el de mi familia.

Gracias a mi padre y a mi abuela, por esos paquetes de comida, de aceite y jamón, tan cargados de cariño y amor. Gracias a mi tía y a mi prima Julia, por todas las risas que le sacan a esta asperger. Gracias a mi tío y a mi abuela, por creer siempre en mí y lucirme con orgullo. Os quiero y veo a todos mucho menos de lo que me gustaría.

A mi casa, a Pedro padre, Pedro chico, a Laura y a mi Madre, por todas las videollamadas de Bon día, de cómo estás, de ver qué cocinaba el canal Huevo Frito. Por vuestro apoyo y cariño, eterno e incombustible. Ya estoy aquí.

Y a mí, coño. Por ser una cabezona de *cuidao*, trabajadora y con algo de disciplina, que ha conseguido acabar esto. Gracias por poner la energía y el trabajo en creer en ti. Tú a tu ritmo.

Next.

8. APPENDIX

APPENDIX I – List of publications

- Lisowski, P., Lickfett, S., Rybak-Wolf, A. **Menacho C.**, et al. Mutant huntingtin impairs neurodevelopment in human brain organoids through CHCHD2-mediated neurometabolic failure. *Nature Communications* (2024). doi: <https://doi.org/10.1038/s41467-024-51216-w>
- Le, S., **Menacho C.**, Prigione A. Balancing neuronal activity to fight neurodevelopmental disorders. *Trends in Neuroscience* (2024). doi: 10.1016/j.tins.2024.03.002.
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- Lickfett, S., **Menacho, C.**, Zink, A., Telugu, N. S., Beller, M., Diecke, S., Cambridge, S., & Prigione, A. High content analysis of neuronal morphology in human iPSC-derived neurons. *STAR Protocols* (2022) doi: <https://doi.org/10.1016/j.xpro.2022.101567>
- Le, S., Petersilie, L., Inak, G., **Menacho-Pando, C.**, Kafitz, K. W., Rybak-Wolf, A., Rajewsky, N., Rose, C. R., & Prigione, A. Generation of Human Brain Organoids for Mitochondrial Disease Modeling. *Journal of Visualized Experiments* (2021) doi: <https://doi.org/10.3791/62756>
- Menacho, C.**, & Prigione, A. Tackling mitochondrial diversity in brain function: from animal models to human brain organoids. *The International Journal of Biochemistry & Cell Biology* (2020) doi: <https://doi.org/10.1016/j.biocel.2020.105760>
- Eraso-Pichot, A., Brasó-Vives, M., Golbano, A., **Menacho, C.**, Claro, E., Galea, E., & Masgrau, R. GSEA of mouse and human mitochondriomes reveals fatty acid oxidation in astrocytes. *Glia* (2018) doi: <https://doi.org/10.1002/glia.23330>

PREPRINTS

- Menacho C**, Okawa S, Álvarez-Merz I, Wittich A, Muñoz-Oreja M, Lisowski P, Pentimalli TM, Rybak-Wolf A, Inak G, Zakin S, Thevandavakkam M, Petersilie L, Zaliani A, Mlody B, Seibt A, Donnelly J, Woleben K, Fernandez-Checa J, Herebian D, Mayatepek E, Rajewsky N, Spinazzola A, Schuelke M, Perlstein E, Rossi A, Distelmaier F, Holt IJ, Pless O, Rose CR, Del Sol A, and Prigione A. Deep learning-driven neuromorphogenesis screenings identify repurposable drugs for mitochondrial disease. *BioRxiv* (2024) doi: <https://doi.org/10.1101/2024.07.08.602501>
- Dalbeyler, S., Herud, A., Zglinicki, B., Ziuzia, P., Piechota, M., Hoinkis, D., Bergauer, L., **Menacho Pando, C. M.**, Soltys, Z., Hanus, P., Groves, P., Dixon, L., Ganglberger, F., Del Prete, D., Dubovyk, V., Abfal, M., Violet, C., Lawless, N., Kielbasa, S. M., ... Slezak, M. Glucocorticoid receptors mediate reprogramming of astrocytes in depression. *BioRxiv* (2025) doi: <https://doi.org/10.1101/2025.04.04.646549>
- Golbano A, Pardo L, **Menacho C**, Rierola M, Claro E, Wood LB, Masgrau R, Galea E. Defective neuritogenesis in Abcd1/2 deficient rat neurons due to intrinsic and astrocyte/dependent mechanisms. *BioRxiv* (2022). doi: <https://doi.org/10.1101/2022.09.30.510337>

APPENDIX II – Patent declaration

This is to declare that two patent applications have been submitted based on parts of the work presented in this dissertation. The applications cover novel aspects of the findings described in the Discussion of this thesis and are intended to protect the intellectual property developed during the course of this doctoral research.

Patent Application 1:

Title of the invention:

Talarozole for the treatment of Leigh syndrome

Application number:

EU application 24163953.3.

Year of submission:

2024

Applicants / Inventors:

Carmen Maria Menacho Pando, Satoshi Okawa, Antonio del Sol, Alessandro Prigione

Patent Application 2:

Title of the invention:

Sertaconazole for the treatment of Leigh syndrome

Application number:

U.S. provisional patent application No. 63/491,017

Year of submission:

2024

Applicants / Inventors:

Carmen Maria Menacho Pando, Alessandro Prigione, Ethan Perlstein, Justin Donnelly, Mathura Thevandavakkam.

These patent applications are under review and are aimed at protecting the innovative contributions made through this doctoral project. Disclosure and publication of related results in this thesis were made in compliance with the rules of the filing process.

APPENDIX III – Affidavit

I, Carmen Maria Menacho Pando, hereby declare under oath that I have produced my thesis independently and without any undue assistance by third parties under consideration of the 'Principles for the Safeguarding of Good Scientific Practice at Heinrich Heine University Düsseldorf'. Any excerpts or illustrations taken from other works, either verbatim or in substance, have been identified as such.

This thesis has not been submitted, either in whole or in part, to any other examination body for the purpose of obtaining an academic degree.

APPENDIX IV – CURRICULUM VITAE

CARMEN MENACHO

RESEARCH EXPERIENCE

PHD STUDENT

2020-PRESENT

Stem Cell Metabolism Lab, University Clinic Düsseldorf
Heinrich Heine University, Düsseldorf, Germany

Group led by Dr. Alessandro Prigione
Project: development of drug discovery pipelines using engineered patient-derived neural cells

- Established high-throughput imaging analysis for compound testing in iPSC-derived neurons
- Developed phenotypical validation in midbrain organoids

RESEARCH ASSOCIATE

2017-2019

Brain Microcircuits in Psychiatric diseases, BioMedX,
Heidelberg, Germany

Group led by Dr. Michał Ślęzak
Project: Unraveling the contribution of astrocyte-specific signaling to the central effects of chronic stress

- Performed single-cell sequencing of isolated adult astrocytes from mouse model of chronic social defeat stress
- Discovered a circadian-dependent signature in astrocytes
- Designed and managed project; communication to academic and industrial sponsors (*Boehringer Ingelheim*)

MASTER THESIS

2016-2017

Laboratory of astrocyte plasticity, Institut de Neurociències,
Universitat Autònoma de Barcelona

Group led by Dr. Elena Galea & Dr. Roser Masgrau
Project: Early astrocyte and neuron dysfunction in X-linked adrenoleukodystrophy.

RESEARCH INTERNSHIP

SUMMER 2016

Laboratory of Glia Biology, VIB-KU Leuven, Belgium

Group led by Dr. Matthew Holt
Project: A novel tool for genetic manipulation of astrocytes

BACHELOR THESIS

2015-2016

Laboratory of astrocyte plasticity, Institut de Neurociències,
Universitat Autònoma de Barcelona

Group led by Dr. Elena Galea & Dr. Roser Masgrau
Project: Role of CREB transcription factor in astrocytes



18 / 08 / 1993



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EDUCATION

PhD Neuroscience

2021 Present Heinrich Heine University
Düsseldorf, Germany

MSc Neuroscience

2016 2017 Universitat Autònoma de
Barcelona, Spain

Year abroad (Erasmus+)

2013 2014 Università degli Studi di
Trieste, Italy

BSc Biomedical Sciences

2011 2015 Universitat Autònoma de
Barcelona, Spain

TEACHING EXPERIENCE

2020 2024 Master Translational
Neuroscience / Module 3a

Heinrich Heine University
Düsseldorf, Germany

2020 2024 Master Molecular
Biomedicine / Module M1530

Heinrich Heine University
Düsseldorf, Germany

QUALITIES



Creative and critical thinking



Time and project management



Self-driven, goal-oriented



Effective communication

LANGUAGES



Native



Proficient



Proficient



B1



A2

TECHNICAL SKILLS

STEM CELL CULTURE AND DIFFERENTIATION

Regular culture iPSC, differentiation into neural progenitors, NGN2 neurons, dopaminergic neurons, cortical organoids, midbrain organoids.

TISSUE PREPARATION

Experienced with dissociation of adult mouse brain tissue and brain organoid tissue for magnetic cell sorting, single cell sequencing and related OMICS.

HISTOLOGY AND IMAGING

Tissue preparation (sectioning with vibratome and cryotome), staining and imaging (fluorescence, confocal) of brain and spinal cord of rodents and stem-cell-derived organoids

IMAGE ANALYSIS

High content imaging analysis using Cell Profiler; basic knowledge of Image J and IMARIS software.
Basic data curation with R programming.

MOLECULAR AND BIOCHEMICAL ASSAYS

Experienced in routine molecular assays (PCR, qPCR,...)
Library preparation for Next Generation sequencing.

CONFERENCES AND MEETINGS

Talk at International Society for Stem Cell Research	2024
Hamburg, Germany	
Poster at International Brain Research Organization	2023
Granada, Spain	
Poster at StemCell Network International Meeting	2023
Aachen, Germany	
Poster at StemCell Network Internal Meeting	2022
Herne, Germany	
Poster at MitoNice	2022
Nice, France	

REFERENCES

-  **Dr. Alessandro Prigione**
alessandro.prigione@med.uni-duesseldorf.de
-  **Dr. Michał Ślęzak**
michal.slezak@port.lukasiewicz.gov.pl

TRAINING AND LICENSES

- 2024 **Career planning**
Impulsplus, Germany
- 2022 **Conflict resolution**
Institut für Internationale Kommunikation, Germany
- 2022 **Project Management in Science**
Brain4hire, Germany
- 2021 **Introduction to OMERO & confocal microscopy**
Center for Advanced Imaging (CAI), Düsseldorf, Germany
- 2021 **Good Scientific Practice**
Heinrich Heine University Düsseldorf, Germany
- 2018 **Introduction to Cytoscape**
EMBL Heidelberg, Germany
- 2016 **FELASA B**
KU Leuven University, Belgium

PATENTS AND AWARDS

- 2024 **Talarozole for the treatment of Leigh syndrome**
EU application 24163953.3.
- 2024 **Sertaconazole for the treatment of Leigh syndrome**
U.S. provisional patent application No. 63/491,017
- 2024 **Travel award winner**
International Society for Stem Cell Research (ISSCR)
- 2024 **Merit award winner**
International Society for Stem Cell Research (ISSCR)

PUBLICATIONS

1. Lisowski, P., Lickfett, S., Rybak-Wolf, A. **Menacho C.**, et al. *Mutant huntingtin impairs neurodevelopment in human brain organoids through CHCHD2-mediated neurometabolic failure.* Nature Communications (2024). doi: <https://doi.org/10.1038/s41467-024-51216-w>
2. Le, S., **Menacho C.**, Prigione A. *Balancing neuronal activity to fight neurodevelopmental disorders.* Trends in Neuroscience (2024). doi: [10.1016/j.tins.2024.03.002](https://doi.org/10.1016/j.tins.2024.03.002).
3. Edwards-Faret G, de Vin F, Slezak M, Gollenbeck L, Karaman R, Shinmyo Y, Batiuk MY, **Pando CM**, Urschitz J, Rincon MY, Moisyadi S, Schnütgen F, Kawasaki H, Schmucker D, Holt MC. *A New Technical Approach for Cross-species Examination of Neuronal Wiring and Adult Neuron-glia Functions.* Neuroscience. (2023) doi: [10.1016/j.neuroscience.2022.11.029](https://doi.org/10.1016/j.neuroscience.2022.11.029).
4. Lickfett, S., **Menacho, C.**, Zink, A., Telugu, N. S., Beller, M., Diecke, S., Cambridge, S., & Prigione, A. *High-content analysis of neuronal morphology in human iPSC-derived neurons.* STAR Protocols (2022) doi: <https://doi.org/10.1016/j.xpro.2022.101567>
5. Le, S., Petersilie, L., Inak, G., **Menacho-Pando, C.**, Kafitz, K. W., Rybak-Wolf, A., Rajewsky, N., Rose, C. R., & Prigione, A. *Generation of Human Brain Organoids for Mitochondrial Disease Modeling.* Journal of Visualized Experiments (2021) doi: <https://doi.org/10.3791/62756>
6. **Menacho, C.**, & Prigione, A. *Tackling mitochondrial diversity in brain function: from animal models to human brain organoids.* The International Journal of Biochemistry & Cell Biology (2020) doi: <https://doi.org/10.1016/j.biocel.2020.105760>
7. Eraso-Pichot, A., Brasó-Vives, M., Golbano, A., **Menacho, C.**, Claro, E., Galea, E., & Masgrau, R. *GSEA of mouse and human mitochondriomes reveals fatty acid oxidation in astrocytes.* Glia. (2018) doi: <https://doi.org/10.1002/glia.23330>

PREPRINTS

1. **Menacho C.**, Okawa S, Álvarez-Merz I, Wittich A, Muñoz-Oreja M, Lisowski P, Pentimalli TM, Rybak-Wolf A, Inak G, Zakin S, Thevandavakkam M, Petersilie L, Zaliani A, Mlody B, Seibt A, Donnelly J, Woleben K, Fernandez-Checa J, Herebian D, Mayatepek E, Rajewsky N, Spinazzola A, Schuelke M, Perlstein E, Rossi A, Distelmaier F, Holt IJ, Pless O, Rose CR, Del Sol A, and Prigione A. *Deep learning-driven neuromorphogenesis screenings identify repurposable drugs for mitochondrial disease.* BioRxiv (2024) doi: <https://doi.org/10.1101/2024.07.08.602501>
2. Golbano A, Pardo L, **Menacho C.**, Rierola M, Claro E, Wood LB, Masgrau R, Galea E. *Defective neuritogenesis in Abcd1/2 deficient rat neurons due to intrinsic and astrocyte/dependent mechanisms.* BioRxiv (2022). doi: <https://doi.org/10.1101/2022.09.30.510337>



