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m.3243A > G-Induced Mitochondrial Dysfunction Impairs Human Neuronal Development and Reduces Neuronal Network Activity and Synchronicity

Graphical Abstract

Highlights
- High m.3243A > G heteroplasmy leads to mitochondrial dysfunction in human neurons
- High heteroplasmy reduces synapses, mitochondria, and dendritic complexity
- High heteroplasmy leads to reduced single-cell neuronal activity
- High heteroplasmy leads to lower neuronal network activity and synchronicity

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In Brief
Using human-inducible-pluripotent-stem-cell-derived neurons with high levels of m.3243A > G heteroplasmy, Klein Gunnewiek et al. show neuron-specific mitochondrial dysfunction as well as structural and functional impairments ranging from reduced dendritic complexity and fewer synapses and mitochondria to reduced neuronal activity and impaired network synchronicity.
m.3243A > G-Induced Mitochondrial Dysfunction Impairs Human Neuronal Development and Reduces Neuronal Network Activity and Synchronicity

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SUMMARY

Epilepsy, intellectual and cortical sensory deficits, and psychiatric manifestations are the most frequent manifestations of mitochondrial diseases. How mitochondrial dysfunction affects neural structure and function remains elusive, mostly because of a lack of proper in vitro neuronal model systems with mitochondrial dysfunction. Leveraging induced pluripotent stem cell technology, we differentiated excitatory cortical neurons (iNeurons) with normal (low heteroplasm) and impaired (high heteroplasm) mitochondrial function on an isogenic nuclear DNA background from patients with the common pathogenic m.3243A > G variant of mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS). iNeurons with high heteroplasm exhibited mitochondrial dysfunction, delayed neural maturation, reduced dendritic complexity, and fewer excitatory synapses. Micro-electrode array recordings of neuronal networks displayed reduced network activity and decreased synchronous network bursting. Impaired neuronal energy metabolism and compromised structural and functional integrity of neurons and neural networks could be the primary drivers of increased susceptibility to neuropsychiatric manifestations of mitochondrial disease.

INTRODUCTION

Mitochondrial disease is caused by mutations in nuclear or mitochondrial DNA (mtDNA). The resulting cellular/tissue energy crisis affects organs with the highest energy need, such as the brain (El-Hattab et al., 2015). Epilepsy, intellectual and cortical sensory deficits, and psychiatric manifestations are the most frequent manifestations of any mitochondrial disease (Andreazza et al., 2018; Finsterer, 2009; Gorman et al., 2016; Kim et al., 2019; Pei and Wallace, 2018; Reinhart and Nguyen, 2019; Srivastava et al., 2018; Sullivan et al., 2018; Nierenberg et al., 2018). Neural processes with high energy demand, such as neuronal maturation/development and plasticity, as well as impaired synaptic physiology and synchronous neuronal activity (Alves et al., 2014; Boku et al., 2018; Quinn et al., 2018; Reinhart and Nguyen, 2019; Serafini, 2012) could explain the proximal neuropsychological presentation in mitochondrial disease. To date, however, the lack of translational model systems of impaired brain bioenergetics has hampered our understanding of the exact nature of disease pathobiology and the development of disease-modifying therapies.

Mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS) is the most common progressive and devastating multisystem mitochondrial disease, with epilepsy, stroke-like episodes, intellectual and cortical sensory deficits, psychopathology, muscle weakness, cardiomyopathy, and/or diabetes (El-Hattab et al., 2015). The majority of MELAS patients (80%) have an adenine-to-guanine pathogenic variant at the m.3243 position (m.3243A > G) of the mitochondrial genome (mtDNA) in the MT-TL1 gene coding for tRNAleu(UUR)
This affects amino acid incorporation during translation of 13 mtDNA proteins essential for oxidative phosphorylation (Sarasar et al., 2008). The estimated prevalence of clinically affected individuals with the m.3243A > G variant causing MELAS is about 1:20,000 (Chinnery et al., 2000; Hirano and Pavlakis, 1994; Majamaa et al., 1998; Manwaring et al., 2007), but that of asymptomatic carriers could be as much as 1:400 in the general population (Manwaring et al., 2007). The percentage of mutated copies of mtDNA (heteroplasmy) plays a role in the onset and expression of symptoms as well as the severity of the disease (Schon et al., 2012). Specifically, levels of heteroplasmy for the m.3243A > G variant positively correlates with mitochondrial respiratory chain complex I, III, and IV insufficiencies (Ciafaloni et al., 1992; Kobayashi et al., 1990, 1991; Ylikallio and Suomalainen, 2012; Yokota et al., 2015). m.3243A > G variant-related phenotypes are highly variable (Hämäläinen et al., 2013). Lower heteroplasmy levels of ~30% commonly present with type I or II diabetes with or without hearing loss, whereas 50% to 80% can present with myopathy, cardiomyopathy, cortical sensory deficits, and psychiatric symptoms and MELAS (Pei and Wallace, 2018; Pia and Lui, 2018; Wallace, 2018). Homoplasmic cases are associated with severe early-onset MELAS or with encephalopathy, including intellectual disability. Although brain heteroplasmy levels in post-mortem brain tissue of individuals with MELAS (Betts et al., 2006) have been correlated with (sub)cortical volume loss (Haast et al., 2018), we know little about the effect of various m.3243A > G heteroplasmy levels on neuronal development and function.

The polyploid nature of mtDNA and replicative segregation hamper the development of animal or in vitro disease models for mtDNA-related mitochondrial diseases (Prigione, 2015). Current in vitro models, such as cytoplasmic hybrids, do not take into account the interplay between patient mtDNA and nuclear genome. For all experiments, iNeurons were co-cultured on freshly isolated rodent astrocytes to facilitate neuronal maturation and, therefore, were sensitive to cytosine arabinoside (Ara-C) treatment. We reprogrammed fibroblasts of an individual with MELAS to iPSCs by retroviral transduction with the Yamanaka transcription factors (see STAR Methods for patient description; Table S1). We selected 5 clones to determine the m.3243A > G heteroplasmy levels by next-generation sequencing. We identified two clones with 0%, two clones with 71%, and one clone with 83% heteroplasmy, a similar spread found in studies using mtDNA heteroplasmy in iPSCs (Hämäläinen et al., 2013; Kodaira et al., 2015; Yang et al., 2018). We selected clones with low heteroplasmy (LH1; 0% m.3243A > G) and high heteroplasmy (HH1; 71% m.3243A > G) for further investigations with the goal of using isogenic clones. Clones with and without m.3243A > G heteroplasmy showed positive expression of the pluripotency markers OCT4, NANOG, SOX2, and LIN28 (Figures S1A and S1B) and normal karyotypes (Figure S1C). We included a curated healthy iPSC line (control [CTR]; 409-B, Kyoto University; Kondo et al., 2017a) in our study to serve as an external CTR to counter any potential bias of the isogenic CTR (LH1) patient background. When we quantified iPSC growth rate (Figure S1D), we observed more cell death after plating and a small reduction in growth rate for HH1 iPSCs compared with CTR and LH1 iPSCs at initial culturing (P0) and after 15 subsequent passages (P+15). Next we differentiated the CTR, LH1, and HH1 iPSCs into a homogeneous population of excitatory cortical layer 2/3 neurons (hereafter referred to as iNeurons) by forced induction and, therefore, were sensitive to cytosine arabinoside (Ara-C) treatment. We quantified the final number of surviving MAP2-positive iNeurons, which formed Synapsin 1/2-expressing synapses, within 23 days in vitro (DIV) of the start of differentiation (Figure 1A; Figure S1E). We observed that differentiation induced higher mortality in the HH1 line, presumably because more cells remained mitotic after Ngn2 induction and, therefore, were sensitive to cytosine arabinoside (Ara-C) treatment. We quantified the final number of surviving MAP2-positive iNeurons (Figure S1F) and adjusted initial plating numbers to obtain the same amount of MAP2 cells after differentiation at DIV 23, the time point when most experiments were performed (Figure S1G; STAR Methods). Importantly, droplet digital PCR (dPCR) showed that heteroplasmy levels were retained across at least 15 iPSC passages, and although we observed a slight decrease post-neuronal differentiation,
Figure 1. m.3243A > G Heteroplasmy Levels per Cell Type and Neuronal Aerobic Metabolic Profiles

(A) Schematic representation of MELAS iPSCs and derived neurons. Shown are patient fibroblast-generated iPSC clones with homoplasmic (0%) or heteroplasmic (71%) mutation levels. Ngn2- and rTta- construct transduction led to 0% and 65% heteroplasmy levels. Subsequently, doxycycline-induced Ngn2 expression mediated the differentiation into iNeurons, confirmed by expression of microtubule associated protein 2 (MAP2) and Synapsin 1/2 at 23 days in vitro (DIV) (scale bars, 100 µm).

(B) Quantification of percent m.3243A > G heteroplasmy upon reprogramming of fibroblasts to iPSCs as well as during Ngn2-dependent differentiation (n = 2–5 per line, per time point).

(legend continued on next page)
we confirmed stable heteroplasmy levels during neuronal maturation (>60%; Figure 1B).

A High Level of m.3243A > G Heteroplasmy Reduces the Mitochondrial Oxygen Consumption Rate

Neuronal differentiation induces a metabolic shift from predominantly glycolytic iPSCs (Prigione et al., 2014) to mitochondrial oxidative phosphorylation (OXPHOS)-dependent neurons (Zheng et al., 2016). We assessed the effects of the m.3243A > G variant on mitochondrial respiration in CTR, LH1, and HH1 iPNeurons with the Seahorse XF Cell Mito stress test at DIV 23. We used oxygen consumption rate (OCR) as a measure of mitochondrial respiration (Figure 1C; Figures S2A and S2B) and extracellular acidification rate (ECAR) as a measure of glycolytic capacity (Figure S2C). We normalized the OCR/ECAR to (1) to the cell count to prevent any bias because of any differences in neuronal cell density that might arise from differences in cell viability development (Figures S1F and Figure S2D) and (2) oxaloacetate-induced citrate synthase (CS) activity (Rodenburg, 2011) to determine OCR per mitochondrion. We obtained comparable results when the OCR was normalized to cell count or CS activity (Figure S2B). CTR and LH1 iPNeurons exhibited similar basal OCR profiles (Figures 1C–1G), whereas HH1 iPNeurons showed a lower basal OCR in comparison. Addition of the ATP-synthase inhibitor oligomycin reduced ATP-linked respiration (Figure 1E) in HH1 iPNeurons compared with CTR and LH1 iPNeurons. Furthermore, the uncoupling agent p-trifluoromethoxy carbonyl cyanide phenyl hydrazone (FCCP) showed a significantly reduced maximal respiratory capacity in HH1 iPNeurons (Figure 1F). Although the OCR was decreased in HH1 iPNeurons under these multiple conditions, we observed an increase in ECAR in HH1 iPNeurons, reflecting an increase in anaerobic glycolysis (Figure 1G; Figure S2F), which was not accompanied by a significant increase in lactate in the medium of HH1 iPNeurons compared with the medium of LH1 iPNeurons (Figure S2J). Subsequently we measured OCR and ECAR in CTR, LH1, and HH1 iPSCs (Figures S2E and S2F). We observed more variation in OCR levels and found no significant differences in basal OCR and ATP-linked OCR or in ECAR or medium lactate levels. We did observe a significant reduction in the maximal OCR of iPSC-HH1 compared with iPSC-LH1 (Figure S2I). Overall, our data show that, in iPNeurons, a high m.3243A > G mutational load affects mitochondrial OCR by reducing OXPHOS while increasing anaerobic glycolysis. In contrast, iPSCs relying mainly on (an)aerobic glycolysis (Bukowiecki et al., 2014; Prigione and Adjaye, 2010) solely showed a reduction in maximal respiratory capacity.

Structural Differences in iPNeurons with High versus Low Levels of m.3243A > G Heteroplasmy

Mitochondria support important aspects of neuronal development, such as axonal (Spillane et al., 2013) and dendritic branching (Agnihotri et al., 2017). We assessed the effects of the m.3243A > G variant on somatodendritic neuronal structure by sparsely transfecting iPNeurons at DIV 6 with a construct expressing red fluorescent protein (Figure 2A). We imaged and reconstructed 3-dimensionally at least 30 iPNeurons per cell line at DIV 23 (Figure 2B) and quantified the soma size, number of primary dendrites, total and mean dendritic length, number of dendritic nodes, and surface covered by the dendritic trees (Figure 2C). CTR and LH1 iPNeurons did not differ from each other in any of the parameters (Figure 2C). Soma size and primary dendrite counts were similar in all cell lines, whereas we observed shorter dendrites in HH1 iPNeurons compared with CTR and LH1 iPNeurons. Furthermore, we observed a reduction in total dendritic length, number of dendritic nodes, and branchpoints in HH1 iPNeurons. Accordingly, the total surface covered by the dendritic tree, quantified by calculating the “convex hull 2D,” was smaller in HH1 iPNeurons (Figure 2C). Finally, we used Sholl analysis by applying expanding 10-μm rings from the soma to quantify the complexity of the dendritic network close to and distal from the soma (Figure 2D). Sholl analysis confirmed a reduced number of dendritic intersections, shorter dendritic length, and fewer dendritic nodes per Sholl ring in HH1 iPNeurons (Figures 2D and 2E). These observations show that iPNeurons with attenuated mitochondrial function are reduced and of less complex dendritic organization and, thus, present a smaller receptive surface.

Synaptic Density and Axonal Mitochondrial Abundance Are Reduced in iPNeurons with High Levels of m.3243A > G Heteroplasmy

In addition to their role in neuronal growth, mitochondria mediate synapse formation and function, whether postsynaptic at dendrites (Li et al., 2004), at en passant pre-synaptic sites in the axon, or at growth cones (Morriss and Hollenberg, 1993; Smith and Gallo, 2018). Interestingly, mitochondrial absence at the synapse has been linked to increased neurotransmitter release probability (Kwon et al., 2016) as well as to loss of synaptic function (Stowers et al., 2002). Our next goal was to investigate whether the m.3243A > G variant affects the number of synapses in iPNeurons. To this end, we measured the number of synapses by quantifying presynaptic Synapsin 1/2 puncta on MAP2-positive dendrites (Figure 3A). Although we found no differences between CTR and LH1 iPNeurons, we did observe fewer Synapsin

(C) Oxygen consumption rate (OCR) measurements at basal level and following supplementation with oligomycin (2 μM), FCCP (2 μM), and rotenone and antimycin A (0.5 μM) at DIV23. The assay was done on 10–12 biological replicates per line per run and repeated twice in its totality (for a total of 30–36 samples per cell line). Raw OCR levels were normalized to an CS assay (Figure S2A).

(D) Quantification of basal respiration; n = 10–12.
(E) Quantification of ATP-linked respiration (basal OCR minus oligomycin response) was averaged over three measurements per sample; n = 10–12.
(F) Quantification of maximal respiration (FCCP response) was averaged over three measurements per sample; n = 10–12.
(G) Quantification of extracellular acidification rate (ECAR; represents glycolysis rate) determined during and averaged over the first six recordings (n = 12). Raw ECAR (Figure S2A) is normalized to an CS assay.

Data represent means ± SEM, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.001, one-way analysis of variance with post hoc Bonferroni correction. CTR, LH, and HH iPNeurons were statistically compared by one-way ANOVA. C1, complex 1 (NADH dehydrogenase); C2, complex 2 (succinate dehydrogenase); C3, complex 3 (coenzyme Q, cytochrome c reductase); C4, complex 4 (cytochrome c oxidase); C5, complex 5 (ATP synthase).
Figure 2. Reconstruction of the Dendritic Morphology

(A) Representative fluorescence microscopy images of DsRed-positive iNeurons of CTR, LH, and HH cultures at DIV 23 (scale bars, 30 μm).

(B) Representative somatodendritic reconstructions of CTR iNeurons.

(C) Quantification of soma size, number of primary dendrites, number of dendritic nodes, mean and total dendritic length, and size of the surface covered by the dendritic network (convex hull 2D); *p < 0.05, ***p < 0.001, one-way ANOVA.

(D) Sequential 10-μm rings placed from the center soma outward for Sholl analysis.

(E) Quantification per 10-μm Sholl section of the number of dendritic intersections per ring, total dendritic length per ring, and number of dendritic nodes per ring. CTR, n = 37; LH, n = 36; HH, n = 35. Data represent means ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.001, two-way ANOVA with post hoc Bonferroni correction.
Figure 3. Quantification of Mitochondrial and Synaptic Density

(A and B) Representative light fluorescence images of CTR, LH, and HH iNeurons (63x magnification) stained with microtubule-associated protein 2 (MAP2) and Synapsin 1/2 (A; scale bars, 30 µm) and quantification of the number of pre-synaptic Synapsin 1/2 puncta per micrometer of MAP2-positive dendritic length (legend continued on next page).
1/2 puncta in HH1 iNeurons compared with CTR or LH1 iNeurons (Figures 3A and 3B) at DIV 23.

Next we quantified the axonal mitochondrial abundance in the proximal part of the axon (30–200 μm from the soma). Using a DsRed2-Mito7 marker, we visualized the entire mitochondrial network of single iNeurons and found that HH1 iNeurons had fewer mitochondria present in the initial part of the axon (30–200 μm from the soma) (Figures 3C and 3D). Assessing mitochondrial morphology, we found no differences in average size, interconnectivity, or shape (Figure S3A), but we did observe an increased proportion of larger and rounder mitochondria in HH1 iNeurons (Figures 3E and 3F).

Depending on species and neuronal subtype, mitochondria can be present in pre-synaptic sites (50% in human pyramidal neurons [Kwon et al., 2016], 82% in rat retinal ganglion neurons [Fischer et al., 2018], 43%–and 56% in mouse hippocampal neurons [Obashi and Okabe, 2013]), where their presence can modify synaptic neurotransmitter release probability (Kwon et al., 2016; Werth and Thayer, 1994). To determine the ratio of presynaptic elements co-localizing with mitochondrial iNeurons, we employed dual DsRed2-Mito7 and VGLUT1-VENUS (pre-synaptic vesicular glutamate transporter) transfection (Figure 3G). HH1 iNeurons again showed a reduced number of mitochondria in the distal axon compared with CTR and LH1 iNeurons (Figure 3H), matching our observations in the proximal axon (Figures 3C and 3D). Second, we observed a reduction in VGLUT1 puncta in HH1 iNeurons compared with CTR and LH1 iNeurons (Figure 3H). The absolute number of VGLUT1 puncta that co-localized with mitochondria in the axon was lower in HH1 iNeurons than in CTR and LH1 iNeurons, although the ratio of synapses co-localizing versus not co-localizing with mitochondria was comparable in CTR and LH1 iNeurons. Similarly, no change in this ratio (synapses with versus without mitochondria) was observed when using DsRed2-Mito7 transfection in combination with endogenous Synapsin staining (Figure S3B). This indicates that, although the total numbers of synapses and mitochondria are reduced in HH1 iNeurons, no compensatory mechanism restores the absolute numbers of synapses co-localizing with mitochondria to levels observed in LH1 and CTR iNeurons.

In summary, we observed reduced numbers of mitochondria in the proximal and distal compartments of the axon in HH1 iNeurons, combined with fewer synapses. Although the absolute number of synapses that contain mitochondria is reduced, the ratio of synapses that co-localize with mitochondria versus those that do not is stable across all cell lines.

**Frequency of Spontaneous Excitatory Activity Is Reduced in iNeurons with a High Level of m.3243A > G Heteroplasmy**

Next we studied the effects of m.3243A > G heteroplasmy on neuronal activity at the single-cell level using whole-cell voltage clamp recordings. We recorded spontaneous excitatory postsynaptic currents (sEPSCs) at ~60 mV for all three neuronal lines (Figure 4A) at DIV 23. We observed a decrease in sEPSC frequency (Figures 4B and 4C) but not sEPSC amplitude (Figures 4D and 4E) of HH1 iNeurons compared with CTR and LH1 iNeurons. Looking at the cumulative distribution of sEPSC frequency and amplitude, we found a higher proportion of larger inter-event intervals in HH1 iNeurons compared with CTR and LH1 iNeurons (Figure 4C) but no difference in amplitude distribution (Figure 4E).

To determine whether differences in sEPSC frequency were due to intrinsic neurophysiological differences, we recorded passive and active properties from CTR, LH1, and HH1 iNeurons at DIV 23 (Figure 4F). We observed no quantitative differences between any of the cell lines in resting membrane potential (Figure 4G), capacitance (Figure 4H), or membrane resistance (Figure 4I). Additionally, we found no differences in active properties such as rheobase (Figure 4J), action potential threshold (Figure 4K), action potential amplitude (Figure 4L), inter-spike interval (ISI) of the evoked action potentials (Figure 4M).

Collectively, our data suggest that the high level of m.3243A > G heteroplasmy-induced reduction in sEPSC frequency was caused by a reduction in synaptic density.

**A High Level of m.3243A > G Heteroplasmy Impairs Neuronal Network Activity and Synchronicity**

The previous experiments showed that, at the single-cell level, iNeurons with high m.3243A > G heteroplasmy form fewer synapses and receive less synaptic input. We next investigated whether this reduced synaptic activity also translated into altered activity at the network level. To this end, we examined and compared the spontaneous activity of neuronal networks derived from LH and HH iNeurons growing on micro-electrode arrays (MEAs) at similar densities (Figure S4A). MEA recordings allowed us to non-invasively and repeatedly monitor neuronal network activity (spikes and bursts) through extracellular electrodes located at spatially separated points across iNeuron cultures (Figures 5A–5C). Because of the known heterogeneity between MELAS patients carrying the m.3243A > G heteroplasmy and to avoid a potentially mediating effect of the patient’s specific genetic background, we generated two additional sets of isogenic MELAS iPSC lines (LH2+3 and HH2+3; characterized previously by Perales-Clemente et al., 2016). We selected clones (B) CTR, n = 21; LH, n = 20; HH, n = 20) at DIV 23. Data represent means ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, one-way ANOVA with post hoc Bonferroni correction.

(C and D) Light fluorescence images of CTR, LH, and HH iNeurons (40× magnification) transfected using the DsRed2-Mito7 construct (C; scale bars, 30 μm) as well as quantification of the number and shape of mitochondrial particles in the initial proximal 200-μm axon section (30 μm from soma to exclude the axon-initial segment) (D; CTR, n = 23; LH, n = 21; HH, n = 21).

(E and F) Average and cumulative (E) mitochondrial size and (F) shape. ***p < 0.001, Kolmogorov-Smirnov test.

(G and H) Light fluorescence images of CTR, LH, and HH iNeurons co-transfected with a DsRed2-Mito7 (mitochondria) and a VGLUT1-VENUS (VGLUT1 puncta) construct (G; scale bars, 30 μm) and quantification of the number of mitochondria, number of VGLUT1 puncta, absolute number of co-localizing (mitochondria plus VGLUT1) puncta, and ratio of co-localization (co-localizing/non-co-localizing) puncta (H; CTR, n = 23; LH, n = 27; HH, n = 21). Data represent means ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, one-way ANOVA with post hoc Bonferroni correction.
Figure 4. Reduced Spontaneous Excitatory Activity in HH iNeurons

(A) Representative electrophysiological traces showing spontaneous excitatory postsynaptic currents (sEPSCs) recorded at −60 mV in iNeuron cultures at DIV 23 (CTR, n = 12; LH, n = 17; HH, n = 13).

(B–E) Quantification of sEPSC frequency (B; including cumulative inter-event interval; C) and sEPSC amplitude (D; including cumulative sEPSC amplitude; E).

(F) Representative firing patterns of CTR, LH1, and HH1 iNeurons, recorded using current clamp whole-cell recording at DIV 21.

(G–I) Quantification of passive membrane properties, including (G) resting membrane potential (Vamp), (H) membrane capacitance (Cm), and (I) membrane resistance.

(J–M) Quantification of step depolarization-evoked action potential (AP) active properties of iNeurons, including (J) rheobase, (K) AP threshold, (L) maximum action potential amplitude, and (M) inter-spike interval (ISI; seconds).

Data represent means ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, one-way ANOVA with post hoc Bonferroni correction.
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A cell image with labels for spikes and bursts.

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C Control

D Low Heteroplasm 1 (LH1)

E Low Heteroplasm 2 (LH2)

F Low Heteroplasm 3 (LH3)

G Mean firing rate (Hz)

H Percentage of random spikes

I Mean bursts / min

J Network bursts / min

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with LH (LH2+3, 0% m.3243A > G) and with HH (HH2+3, 66%–84% m.3243A > G; Figure S4B) and differentiated these into iNeurons.

During the fifth week in vitro (DIV 30), all CTR networks (i.e., CTR, LH1, LH2, and LH3) at similar density (Figure S4A) showed a pattern of activity characterized by regular synchronous events called network bursts (Figures 5C–5F). These network bursts are an important characteristic of a properly developed mature neuronal network (Frega et al., 2017). At this stage, we observed no difference in the level and pattern of synchronous activity between the CTR and LH1-3 networks (Figures 5C–5J). The highly reproducible network characteristics observed across all CTR and LH1-3 lines provided us with a consistent and robust standard with which we could directly compare the HH1-3 networks.

iNeurons with high levels of m.3243A > G heteroplasmy (HH1–HH3) showed spontaneous activity with bursts (Figures 5D–5F) of a relatively similar duration (Figure S4C) and comparable number of spikes as iNeurons with LH (Figure S4D, burst firing rate). However, the amount and pattern of spike and network bursting in HH1–HH3 networks were significantly different compared with their respective LH isogenic CTRs. We found that the general level of activity (mean firing rate [MFR]) exhibited by the HH1–HH3 networks was strongly reduced (Figure 5G). Furthermore, HH1–HH3 networks presented with a reduced network burst rate (NBR) (Figure 5I), with HH1 and HH3 networks exhibiting virtually no network bursts (Figures 5D, 5F, and 5I). Network burst duration (NBD) in HH2 and HH3 networks, however, was not affected (Figure S4F). Notably, because network bursts were very sparse in HH3 networks, we were unable to calculate NBDs (Figure S4F) or network inter-burst intervals (NIBI) for HH3 (Figure S4G). Finally, we also observed that spike organization in HH1–HH3 networks differed from CTRs, as indicated by the higher percentage of random spikes (PRS) occurring outside of the network bursts (Figure 5H). Taken together, these results show that iNeurons with high levels of m.3243A > G heteroplasmy fail to organize into functional neuronal networks and produce a distinctive phenotypical pattern of network activity.

Next we assessed whether and to what degree CTR and LH networks differed from HH networks when taking all measured network parameters into account. To this end, we performed a canonical discriminant function analysis, including MFR (Figure 5G), PRS (Figure 5H), mean burst rate (MBR; Figure 5I), mean burst duration (MBD; Figure S4C), mean burst firing rate (MBFR; Figure S4D), mean burst interval (MBI; Figure S4E), NBR (Figure 5I), NBD (Figure S4D), and NIBI (Figure S4G) as variables (Figure S4H). The analysis revealed that HH groups clearly clustered together outside of the CTR/LH spectrum (Figure S4I). Based on this model, we could not only predict an individual’s membership in the larger known group but also cluster them into the respective subgroups (Figure S4I). Combined, the data show that impaired energy metabolism in iNeurons affects neuronal network organization and activity, resulting in a distinct neuronal network phenotype.

Intermediary m.3243A > G Heteroplasmy and Network Activity

Next, to test the outstanding question whether an intermediate level of m.3243A > G heteroplasmy results in intermediate expression of a neuronal phenotype, we recorded the network activity of iNeurons of various densities (Figure S5A) with 30% m.3243A > G heteroplasmy (intermediate heteroplasmy 3 [IH3]) from patient 3 (Figure S5B) at DIV 30 and compared it with LH3 and HH3 (Figures 6A–6D). Overall, IH3 iNeurons fire synchronized network bursts (NBs) comparable with LH3 iNeurons (Figures 6B and 6C) and displayed a similar MFR (Figure 6E), percentage of random activity (Figure 6F), burst rate (Figure 6G), NBR (Figure 6H), as well as other network parameters (Figures S5C–S5F). However, when taking a closer look at the pattern of network activity, IH3 iNeurons exhibited a larger variance in NIBI, measured as the coefficient of variance (CV; Figures 6C and 6J). In other words, there is a larger variation in the intervals between individual NBs, clearly visualized in a cumulative distribution of individual NIBI (Figure S5G).

A Mosaic Co-culture Reveals Distinct Neuronal Network Phenotypic m.3243A > G Thresholds

Finally, to test whether a mosaic co-culture of iNeurons with low and high m.3243A > G heteroplasmy would result in linear or discontinuous expression of the neuronal phenotype, we co-cultured LH1 and HH1 iNeurons at different ratios on MEAs (LH1:HH1 ratio: 100:0, 80:20, 60:40, 40:60, 20:80, and 0:100; Figures 7A–7G).

Interestingly, the firing rate (Figure 7H), burst rate (Figure 7I), and NBR (Figure 7J) in neural networks containing a minimum of 20% LH1 were similar to 100% LH cultures. However, like our observations with IH3 iNeurons, the NIBI coefficient of variance (CV) increased by the presence of 60% HH1 (Figure 7M) or an average heteroplasmy level of 36% (Figure S6A). These results strongly suggest that a continuous change in average m.3243A > G heteroplasmy results in discontinuous (i.e., threshold-dependent) expression of neural network phenotypes, but such heteroplasmy thresholds might be specific to distinct neuronal network phenotypes; e.g., altered NBR at ~60% heteroplasmy versus NIBI already at ~30% heteroplasmy levels.

![Figure 5. Spontaneous Excitatory Network Activity Recorded on MEAs](image-url)
DISCUSSION

Here we report the development and deep phenotyping of a neural model system of mtDNA-related mitochondrial disease. Specifically, we used state-of-the-art iPSC technology (multiple patient lines and leveraging naturally occurring isogenic lines with various levels of mitochondrial dysfunction) combined with in-depth morphological and electrophysiological phenotyping (network and single-cell level) to identify hitherto unknown cell- and network-level neural phenotypes of mitochondrial disease.

iNeurons reprogramming produced clones with different heteroplasmy levels, including homoplasmic clones, because of natural underlying heterogeneity in the original fibroblast population with concomitant changes in respiratory chain activity (this study and Perales-Clemente et al., 2016). This allowed us to use iNeurons with an isogenic nuclear background (eliminating a potential confounding effect because of differences in nuclear genetic background) and with appropriate heteroplasmy levels and respiratory function for disease modeling. We observed that iNeurons generated from individuals with the m.3243A > G variant faithfully replicate brain-specific manifestations of respiratory complex deficiency. iNeurons also revealed clues for understanding the pathomechanism related to abnormal energy metabolism in the brain. Specifically, we found evidence that iNeurons with a high level of m.3243A > G heteroplasmy exhibited reduced dendritic length and complexity. Furthermore, we found a reduction in the number of mitochondria in the proximal and distal sections of the axon combined with a reduction in pre-synaptic protein abundance. On a functional level, iNeurons with high levels of m.3243A > G heteroplasmy were less active at the single-neuron and neuronal network level and showed a reduction in network synchronicity.

Neuropathological studies have expanded our understanding of neural impairment and cell loss in brains of individuals with mitochondrial disease, revealing structural alterations. Mitochondrial dysfunction has been associated with altered neuronal dendritic morphology and remodeling (Tsuyama et al., 2017). Local availability of mitochondrial mass is critical for generating...
Figure 7. Spontaneous Network Activity for Mosaic Co-cultured Neuronal Networks Consisting of 0%–100% LH1 and 0%–100% HH1 iNeurons

(A) Schematic representation of mosaic co-cultured neuronal networks.
(B–G) Example raster plots from recordings of neuronal network activity at DIV 30 of co-cultures containing different ratios of LH and HH iNeurons, as indicated: (B) 100% LH1 + 0% HH1 (n = 19), (C) row 2 with 20% LH1 and 80% HH1 (n = 11), (D) row 3 with 40% LH1 and 60% HH1 (n = 10), (E) row 4 with 60% LH1 and 40% HH1 (n = 9), (F) row 5 with 80% LH1 and 20% HH1 (n = 8), and (G) row 6 with 0% LH1 and 100% HH1 (n = 12). All raster plots represent 3-min representative recordings of each different condition.

(H–M) Quantification of MEA parameters: (H) MFR (hertz), (I) PRS, (J) burst rate (or number of bursts per minute), (K) NBR (or NBs per minute), and (L) and (M) CV of the interval between NBs (percent).

Data represent means ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.001, one-way ANOVA with post hoc Bonferroni correction.
and sustaining dendritic arbors, and disruption of mitochondrial distribution in mature neurons is associated with structural alterations in neurons (Kuzawa et al., 2014; Lopez-Domenech et al., 2016; Spillane et al., 2013). Loss of interneurons (Lax et al., 2016) and synapses and dendritic atrophy (in number and size) in specific brain areas, such as various cortical areas, the cerebellum, thalamus, and basal ganglia (Bettis et al., 2006; Briston and Hicks, 2018; Chen et al., 2017; Cobley, 2018; Quintana et al., 2010; Turnbull et al., 2010) have also been reported in individuals with mitochondrial dysfunction. We found a reduced number of primary dendrites and dendritic nodes, decreased total and mean dendritic length, as well as a reduced surface covered by the dendritic network. We also observed reduced synaptic density in iNeurons with high levels of heteroplasmy, concomitant with a reduction in the number of mitochondria in the proximal and distal section of the axon. Although our culture is restricted to excitatory neurons, our observations corroborate the notion that mitochondrial function and positioning, influencing dendritic branch morphology, play a direct role in establishing and maintaining mature neuronal circuits.

iNeurons with high levels of m.3243A > G heteroplasmy exhibited a strongly reduced MFR at the single-cell and network level. Neuronal activity, being heavily dependent on glucose supply as the main fuel source (Magistretti and Allaman, 2015), is especially vulnerable to metabolic dysregulation. Thus, pathological brain states, such as epilepsy, characterized by firing instability and recurrent seizures, reflecting aberrant synchronous activity of large groups of neurons, are likely to be associated with impaired energy flows in the brain. In a recent study, Styr et al., (2019) observed that metabolic signaling constitutes a core regulatory module of MFR homeostasis. Our results confirm the link between neuronal energy metabolism and MFR homeostasis and corroborate the notion that mitochondrial dysfunction could be causal in initiation and progression of distinct types of epilepsy (Zsurka and Kunz, 2015).

Synchronous rhythms represent a core mechanism for sculpting temporal coordination of neural activity in brain-wide networks (Buzsáki et al., 2013). Common symptoms of m.3243A > G-related mitochondrial disease, such as epilepsy, intellectual and cortical sensory deficits, as well as psychopathology, are also characterized by excessive or asynchronous neuronal activity that typically does not occur in a single isolated neuron; rather, it results from pathological activity in large groups or circuits of neurons (Alexander et al., 2016; Beghi et al., 2005; Leistedt and Linkowski, 2013; Lenartowicz et al., 2018; Wang, 2010). Energy is required to fuel the formation and synchronized activity of neuronal circuits (Jan and Jan, 2010; Spruston, 2008). Altered energy metabolism in the brain, therefore, leads to dramatic changes in the underlying synchronization and functioning of these networks (Quinn et al., 2018; Styr et al., 2019). Impaired mitochondrial structure and function predispose neuronal network dysfunction (Virlogeux et al., 2018). Various in vitro and in vivo model systems have also shown impaired neuronal oscillatory function in mitochondrial disease models (Chan et al., 2016; Kann et al., 2011); for a review, see Chan et al., 2016. Our observations of a reduced level of neuronal network activity as well as the pattern of spiking and the bursting activity of neuronal networks caused by intermediate and HH levels support the notion that neuronal networks with mitochondrial dysfunction fail to synchronize properly. Regulation of synchronous brain activity in individuals with mitochondrial disease could therefore be proximal to the clinical phenotype of epilepsy, cognitive impairment, and neuropsychiatry.

Variation in the percentage of m.3243A > G heteroplasmy in the brain can be associated with phenotypic heterogeneity of neuropsychiatric presentations in MELAS. To determine the basis of this phenotypic heterogeneity, we generated iNeurons with high (60%–80%), low (0%), and intermediate (30%) levels of heteroplasmy as well as neuronal networks compromised of a mosaic of iNeurons with low and high m.3243A > G heteroplasmy (Figure 7A). Both experiments demonstrated that low to intermediate levels of m.3243A > G heteroplasmy did not affect general neuronal network parameters, such as the level of activity or number of network bursts. However, IH to HH levels as well as co-cultures of neuronal networks containing a larger proportion of HH iNeurons did affect the regularity of the NB firing pattern, a phenotype that has been seen in other iPSC-derived models of neurodevelopmental disorders (Frega et al., 2019). Reduced network regularity could also contribute to the epilepsy and stroke-like episodes in MELAS (El-Hattab et al., 2015) and other epilepsy types linked to metabolic dysfunction (Kann et al., 2005; Kudin et al., 2009; Lee et al., 2008) or epileptic animal models (Folbergrová and Kunz, 2012; Folbergrová et al., 2010). Interestingly, these co-cultures of LH and HH neuronal networks demonstrated that the presence of LH, i.e. healthy neurons, could balance the effect of neurons with HH levels on network phenotypes. Furthermore, we observed a rather sharp transition in neuronal network phenotypes in co-cultures consisting of 60% or 80% HH iNeurons, indicating that, when a certain threshold of heteroplasmy level is reached, it cannot be compensated by the presence of LH (healthy) neurons. Similar to our results, a recent study by Picard et al. (2014), using cybrids harboring various levels of m.3243A > G heteroplasmy, showed that small increases in mutant mtDNA caused relatively modest defects in oxidative capacity but resulted in sharp transitions in cellular phenotypes. Our results provide additional evidence that continuous changes in mtDNA heteroplasmy result in discontinuous changes in neuronal network phenotypes. These data also corroborate clinical observation that cases with 60%–80% of m.3243A > G heteroplasmy are associated with severe early-onset MELAS with intellectual and cortical sensory deficits and psychiatric symptoms (Picard et al., 2014; Wallace, 2018).

Taken together, investigating the relationship between impaired brain energy metabolism in a disease-relevant tissue recapitulated similar structural and functional neuronal phenotypes that exist in epilepsy: intellectual and cortical sensory deficits. These results suggest that mitochondrial dysfunction could be a primary driver in initiation and progression of neuropsychiatric symptoms in individuals with mitochondrial disease. These results go beyond the etiology of MELAS disease and provide a conceptual advance in understanding the effect of mitochondrial dysfunction on the structure and function of single neurons and neural networks. Our results not only help us to understand the pathobiology of common neuropsychiatric symptoms in
mitochondrial disease but could be leveraged for future pharmacological studies.

STAR METHODS

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  - Neuronal Differentiation
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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2020.107538.

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AUTHOR CONTRIBUTIONS


DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES


# STAR★METHODS

## KEY RESOURCES TABLE

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**Plasmids (DNA)**

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**Software and Algorithms**

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**Chemicals, Peptides, and Recombinant Proteins**

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RESOURCES AVAILABILITY

Lead Contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, dr. Nael Nadif Kasri (n.nadifkasri@radboudumc.nl).

Materials Availability
Aside from the patient-derived inducible pluripotent stem cell lines, this study did not generate novel unique reagents. The iPSC lines generated in this study can be made available on request, but we may require a payment and/or a completed Materials Transfer Agreement if there is a potential for commercial application.

Data and Code Availability
The authors confirm that the data supporting the findings of this study are available within the article [and/or] its supplementary material. Original data have been deposited to Mendeley Data: https://dx.doi.org/10.17632/crz8f9k9gy.1 and are available from the corresponding author on request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals
For the dissection and culturing of rat astrocytes pregnant WT Wistar WU rats from Charles River were sacrificed after which embryos (E18) were removed for generating primary cultures (see section Primary neuronal cell culture) (Charles River). Animal experiments were conducted in conformity with the Animal Care Committee of the Radboud University Nijmegen Medical Centre, the Netherlands, under DEC application number 2015-0038, and conform to the guidelines of the Dutch Council for Animal Care and the European Communities Council Directive 2010/63/EU.

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Subject #1 was born, as a second child of a then healthy 29-year-old mother, who was later diagnosed with a classic MELAS phenotype, due to 3243A > G mutations. The older sister, who developed severe depression around the age of 32 years, and had recurrent episodes of visual loss, received the same diagnosis after her first stroke-like episode. Our patient had normal growth and adequate early psychomotor development. During puberty he was evaluated for exercise intolerance and fatigue. He was diagnosed with cardiomyopathy at the age of 31 years. He became obese and was diagnosed with type 2 diabetes mellitus. He developed severe, therapy-resistant depression at the age of 33 years. He was found to have homoplasmic 3243A > G mutations in urine sediment cells, 58% heteroplasmic in blood and 89% in fibroblasts. Further metabolic evaluation detected elevated lactic acid levels. Additional BAER test detected unilateral sensorineural hearing loss. At the age of 42 years our patient is using a wheelchair due to progressive muscle weakness and has a mild asymmetry in muscle strength after two stroke-like episodes. His cardiac disease is stable on beta-blocker therapy. His hearing loss became bilateral.

Subject #2 had no family history of mitochondrial disease. Her only notable clinical feature in the pediatric age was a childhood onset progressive sensorineural hearing loss, which needed a left cochlear implant treatment. During puberty she developed insulin dependent Diabetes mellitus and depression. She was also treated for chronic constipation. She had no other health concerns till the age of 36 years, when she had the first of her recurrent stroke like episodes. She also developed seizures and gait ataxia and has a slowly progressive cognitive decline in association with the recurrent stroke like episodes.

Subject #3 had a history of diabetes and deafness in his mother. Her pediatric disease history was uneventful. At the age of 23 years she developed insulin dependent Diabetes mellitus. At the age of 25 years she was diagnosed with hearing loss and had her first episode of major depression. She was also diagnosed with nephropathy and microalbuminuria at the age of 25 years. At the age of 30 years she had the first of her recurrent stroke like episodes. At the age of 35 years she was treated for concentration and memory loss and suicidal ideas. At age 37 she was diagnosed with ophthalmoplegia, ptosis, and myopathy, developed chronic fatigue, exercise intolerance, and in her late 30s peripheral neuropathy. Her MRI showed brain atrophy with subcortical white matter lesions and basal ganglia involvement. At age 45 she was found to have heteroplasmic 3243A > G mutations in 47% in urine sediment cells, 16% heteroplasmia in blood and 33% in fibroblasts.

**Human Fibroblast Donors**

We used iPSCs and/or skin fibroblasts from three distinct subjects, the details of which are included in Table S1. Subject #1 was born, as a second child of a then healthy 29-year-old mother, who was later diagnosed with a classic MELAS phenotype, due to 3243A > G mutations. The older sister, who developed severe depression around the age of 32 years, and had recurrent episodes of visual loss, received the same diagnosis after her first stroke-like episode. Our patient had normal growth and adequate early psychomotor development. During puberty he was evaluated for exercise intolerance and fatigue. He was diagnosed with cardiomyopathy at the age of 31 years. He became obese and was diagnosed with type 2 diabetes mellitus. He developed severe, therapy-resistant depression at the age of 33 years. He was found to have homoplasmic 3243A > G mutations in urine sediment cells, 58% heteroplasmic in blood and 89% in fibroblasts. Further metabolic evaluation detected elevated lactic acid levels. Additional BAER test detected unilateral sensorineural hearing loss. At the age of 42 years our patient is using a wheelchair due to progressive muscle weakness and has a mild asymmetry in muscle strength after two stroke-like episodes. His cardiac disease is stable on beta-blocker therapy. His hearing loss became bilateral.

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**Human iPSC Lines**

Skin fibroblasts of MELAS subject #1 with the pathogenic variant m.3243A > G in MT-TL1 (tRNA^Leu(UUR were reprogrammed by the in house Rudbodumc Stem Cell Therapy Centre, through retroviral transduction of the Yamanaka transcription factors Oct4, c-Myc, Sox2 and Klf4 (Takahashi and Yamanaka, 2006). IPS cells for subject #2 (HH2+3; Table S1) were reprogrammed from primary human skin fibroblasts by ReGen Theranostics (Rochester, MN) using CytoTune-iPSC 2.0 Sendai Reprogramming Kit according to manufacturer’s instructions (Invitrogen A16517) (Perales-Clemente et al., 2016). Previous studies have shown the reprogramming process can generate a large variety of heterogeneity in heteroplasmy levels, in iPSC clones, ranging from 0%–100% (Fujikura et al., 2012; Hämäläinen et al., 2013; Kodaira et al., 2015; Yang et al., 2018). M.3243A > G heteroplasmia levels of 5 iPS clones were sequenced using a PGM Ion Torrent sequencer (ThermoFisher), which showed two clones with 0% m.3243A > G heteroplasmia, two clones with 71% heteroplasmia, and one clone with 83% heteroplasmic. Subsequently, we selected one iPS clone with 0% m.3243A > G heteroplasmia (“Low heteroplasm 1”; LH1), and one IPS clone with 71% m.3243A > G heteroplasmic (“High heteroplasm 1”; HH1) for further testing: we confirmed they both had normal karyotypes (Figure S1C). An additional, curated control cell line (“Control”; CTR; Kondo et al., 2017a; Okita et al., 2011) was included, and confirmed to have no m.3243A > G heteroplasm. This control line was originally derived from fibroblasts of a 36 year-old female, reprogrammed using episomal reprogramming, and showed no karyotypical malformations (Kondo et al., 2017a; Okita et al., 2011). Lines LH2+3 and HH2+3 were generous gifts from Ester Perales-Clemente and Timothy Nelson, previously characterized and tested for heteroplasmy levels (Table S1), and had normal karyotypes (Perales-Clemente et al., 2016). IPS cells were thawed and cultured at all times on recombinant human laminin LN521 (Biolamina, 2021-21), kept in EB basal medium (Thermo Fisher Scientific) supplemented with primocin (0.1 μg/ml, Invivogen), puromycin (0.5 μg/ml) and G418 (0.5 μg/ml), and uridine (50 μg/ml) and incubated at 37°C/5%CO2, with medium changes every 2-3 days and passages 1-2 times per week using ReLeSR (Stem Cell Technologies). Heteroplasmy levels were regularly measured to insure they were retained across passage numbers. All measurements were performed on iPS cells with passage lower than 40.

**Neuronal Differentiation**

IPS cells were directly derived into upper-layer, excitatory cortical neurons by overexpressing the neuronal determinant Neurogenin 2 (Ngn2) upon doxycycline treatment based on Zhang et al. (2013), and as we described previously (Frega et al., 2017). All cells retained their level of heteroplasmy after antibiotic selection for the RITA- and Ngn2 constructs (Figure 1A). RITA/Ngn2-positive IPS cells were plated as single cells at DIV0 onto nitric-acid treated coverslips coated with 50μg/mL poly-L-ornithine hydrobromide (PLO; Sigma-Aldrich #P3655-10MG) and 5 μg/mL human recombinant laminin 521 (BioLamina #LN521-02) in E8 basal medium (GIBCO #A1517001) supplemented with 1% Penicillin/Streptomycin (Pen/Strep; Sigma-Aldrich P4333), 1% RevitaCell (Thermo-Fisher #A2644501), and 4 μg/mL doxycycline (Sigma-Aldrich #D9891-5G) to drive Ngn2 expression, incubated at 37°C/5%CO2, at 20,000 cells per well (24 well plate) for the CTR- and LH- lines, and 30,000 cells per well (24 well plate) for HH lines. At DIV1, medium
was added. The background conversion of DTNB was measured spectrophotometrically at 412 nm and 37°C. The cells were afterward washed 3 times for 5 minutes at room temperature, followed by incubation with secondary antibodies, diluted 1:2000 in blocking buffer, for 1 hour at room temperature. After washing for 3 times for 5 minutes at RT with DPBS, Hoechst (Termofischer #H3570) diluted 1:10.000 in DPBS was incubated for 10 minutes, before adding 4-(trifluoromethoxy) phenylhydrazone FCCP (Sigma #C2920-50MG), and 0.5 μM of antimycin A (Sigma #A8674-25G), respectively. One measuring cycle consisted of 3 minutes of mixing, 3 minutes of waiting and 3 minutes of measuring. The OCR was normalized to citrate synthase activity, to correct for the mitochondrial content of the samples (Rodenburg, 2011). The citrate (CS) activity was measured according to a protocol previously described (Srere, 1969), modified for Seahorse 96 wells plates. We record background citrate synthase activity in the absence of its substrate acetyl-CoA, followed by adding Acetyl-CoA, and subtract the initial activity from the activity in the presence of substrate, providing a measurement of citrate synthase activity. To avoid the possibility that protein levels are similar but mitochondrial count is not, we subsequently normalize mitochondrial respiration to CS. As the deficiency due to the m.3243A>G heteroplasmy should be present in the oxidative phosphorylation chain, not the in the initial phase of the TCA cycle, there is no difference in CS activity. In detail, after completion of OCR measurements the Seahorse medium was replaced by 0.33% Triton X-100, 10 mM Tris-HCl (pH 7.6), after which the plates were stored at −80°C. Before measurements, the plates were thawed and 3 mM acetyl-CoA, 1 mM DTNB, and 10% Triton X-100 were added. The background conversion of DTNB was measured spectrophotometrically at 412 nm and 37°C for 10 minutes at 1-minute intervals, using a Tecan Spark spectrophotometer. Subsequently, the reaction was started by adding 10 mM of the citrate synthase substrate oxaloacetate, after which the synthase activity was calculated from the rate of DTNB conversion in the presence of oxaloacetate, subtracted by the background DTNB conversion rate, using an extinction coefficient of 0.0136 μmol-1. cm-1.

**METHOD DETAILS**

**Droplet Digital PCR (ddPCR) to Measure MT-TL1 m.3243A > G Heteroplasmy**

Droplet digital PCR was performed on DNA extracted from both neurons and iPSC cells. ddPCR primers were custom synthesized to amplify the mitochondrial MT-TL1 m.3243 region. A HEX-labeled hydrolysis probe was designed to detect the wild-type allele (m.3243A), while a FAM-labeled hydrolysis probe was designed against the mutant allele (m.3243G). DNA samples were diluted to ensure proper droplet saturation for an accurate ddPCR quantitative measurement. ddPCR reactions contained 11 ul of 2XddPCR Supermix (no dUTP) (Bio-Rad), 2.2 uL of diluted sample, 1.1 uL of Hind III (NEB), along with 900 nM of PCR primers, 250 nM of each probe and water to a final volume of 22 uL. Samples were loaded into a 96-well plate, heat-sealed with foil, vortexed, centrifuged, and placed on an automated droplet generator (AutoDG, Bio-Rad). After droplet generation, plates were sealed, placed into a thermocycler and PCR was performed with 95°C for 10 min, 40 cycles of denaturation at 94°C for 30 s, annealing and extension at 60°C for 1 min, and a final enzyme deactivation step at 98°C for 10 min. Plates were stored at 4°C until measured on the QX200 droplet reader (Bio-Rad). Data analysis was performed with QuantaSoft Analysis Pro version 1.0.596 (Bio-Rad).

**Seahorse Mito Stress Test**

Oxygen consumption rates (OCR) were measured using the Seahorse XFe96 Extracellular Flux analyzer (Seahorse Bioscience), IPS cells were seeded at a concentration of 10,000 per well in E8 basal medium supplemented with 1% Pen/Strep, 10 μM RevitaCell, and 4 μg/mL doxycycline, and allowed to adhere at 37°C and 5% CO2. A similar maturation protocol was applied as previously described, up until the cells were 23 days in vitro. One hour before measurement, culture medium was removed and replaced by Agilent Seahorse XF Base Medium (Agilent #103334-100) supplemented with 10 mM glucose (Sigma), 1 mM sodium pyruvate (GIBCO), and 200 mM L-glutamine (Life sciences) and incubated at 37°C without CO2. Basal oxygen consumption was measured six times followed by three measurements after each addition of 1 μM of oligomycin A (Sigma #75351-gMG), 2 μM carbonyl cyanide 4-(trifluoromethoxy) phenyl/hydrazone FCCP (Sigma #C2920-50MG), and 0.5 μM of rotenone (Sigma #R8875-25G) and 0.5 μM of antimycin A (Sigma #A8674-100MG), respectively. One measuring cycle consisted of 3 minutes of mixing, 3 minutes of waiting and 3 minutes of measuring. The OCR was normalized to citrate synthase activity, to correct for the mitochondrial content of the samples (Rodenburg, 2011). The citrate (CS) activity was measured according to a protocol previously described (Srere, 1969), modified for Seahorse 96 wells plates. We record background citrate synthase activity in the absence of its substrate acetyl-CoA, followed by adding Acetyl-CoA, and subtract the initial activity from the activity in the presence of substrate, providing a measurement of citrate synthase activity. To avoid the possibility that protein levels are similar but mitochondrial count is not, we subsequently normalize mitochondrial respiration to CS. As the deficiency due to the m.3243A>G heteroplasmy should be present in the oxidative phosphorylation chain, not the in the initial phase of the TCA cycle, there is no difference in CS activity. In detail, after completion of OCR measurements the Seahorse medium was replaced by 0.33% Triton X-100, 10 mM Tris-HCl (pH 7.6), after which the plates were stored at −80°C. Before measurements, the plates were thawed and 3 mM acetyl-CoA, 1 mM DTNB, and 10% Triton X-100 were added. The background conversion of DTNB was measured spectrophotometrically at 412 nm and 37°C for 10 minutes at 1-minute intervals, using a Tecan Spark spectrophotometer. Subsequently, the reaction was started by adding 10 mM of the citrate synthase substrate oxaloacetate, after which the ΔA412 nm was measured again for 10 minutes at 1-minute intervals. The citrate synthase activity was calculated from the rate of DTNB conversion in the presence of oxaloacetate, subtracted by the background DTNB conversion rate, using an extinction coefficient of 0.0136 μmol-1. cm-1.

**Immunohistochemistry**

Neurons were briefly washed with ice-cold DPBS (GIBCO #14190-094), before fixation with 4% paraformaldehyde/ 4% sucrose (v/v), and subsequently permeabilized (DPBS, 0.2% Triton X-100 (Sigma-Aldrich #9002-93-1)). Cells were again washed 3 times with DPBS for 5 minutes at room temperature, before a specific binding of antibodies was prevented by incubation with blocking buffer (DPBS, 5% normal horse serum, 5% normal goat serum, 5% normal donkey serum, 0.1% bovine serum albumine (BSA), 1% glycine, 0.4% triton, 0.1% lysine (all from Sigma-Aldrich)) for 1 hour at room temperature. Primary antibodies were diluted 1:1000 in blocking buffer, and were incubated overnight, at 4°C. The cells were afterward washed 3 times for 5 minutes at room temperature, followed by incubation with secondary antibodies, diluted 1:2000 in blocking buffer, for 1 hour at room temperature. After washing for 3 times for 5 minutes at RT with DPBS, Höchst (Termofischer #H3570) diluted 1:10.000 in DPBS was incubated for 10 mi-
nutes at RT. After one last wash with DPBS for 5 minutes at RT the coverslips were imbedded in fluorescent mounting medium (DAKO #S3023). Primary antibodies that were used, are: mouse anti-MAP2 (1:1000; Sigma M4403), and guinea pig anti-synapsin 1/2 (1:1000; Synaptic Systems 106004). Secondary antibodies that were used, are: goat anti-mouse Alexa Fluor 488 (1:2000, Invitrogen A-11029), and goat anti-guinea pig Alexa Fluor 568 (1:2000, Invitrogen). Imaging was done on a Zeiss Axios Imager Z1 equipped with apotome, using the same settings for all batches and groups, at a resolution of 1024 x 1024 at 63X magnification (scale 1 pixel = 0.072μm). Images were analyzed using ImageJ software (Schneider et al., 2012), whereby iNeurons were selected based on both Map2- and Synapsin expression. Synapsyn-puncta were counted manually and normalized to the length of the dendritic branch on which they reside.

**Neuronal Morphometrical Reconstruction**

To examine morphology of iNeurons, cells on coverslips were transfected with plasmids expressing Discosoma species red (dsRED) fluorescent protein one week after plating. DNAin (MTI-GlobalStem) was used according to manual instructions. Medium was re-freshed completely the day after DNAin application. After three weeks of differentiation cells were fixed in 4% paraformaldehyde/4% sucrose (v/v) in PBS and mounted with DAKO.

**Whole-Cell Patch Clamp Recordings**

Experiments were performed in a recording chamber on the stage of an Olympus BX51WI upright microscope (Olympus Life Science) equipped with infrared differential interference contrast optics, an Olympus LUMPlanFL N 40x water-immersion objective (Olympus Life Science) and kappa MXC 200 camera system (Kappa optronics GmbH) for visualization. Through the recording chamber a continuous flow of carbogenated artificial cerebrospinal fluid (ACSF) containing (in mM) 124 NaCl, 1.25 NaH2PO4, 3 KCl, 26 NaHCO3, 11 Glucose, 2 CaCl2, 1 MgCl2 (adjusted to pH 7.4), warmed to 30°C, was present. Patch pipettes (6-8 MΩ) were pulled from borosilicate glass with filament and fire-polished ends (Science Products GmbH) using the PMP-102 micropipette puller (MicroData Instrument).

For recordings of spontaneous excitatory postsynaptic currents (sEPSCs) in voltage clamp mode, pipettes were filled with a cesium-based solution containing (in mM) 115 CsMeSO3, 20 CsCl, 10 HEPES, 2.5 MgCl2, 4 Na2-ATP, 0.4 Na3-ATP, 10 Na-phosphocreatine, 0.6 EGTA (adjusted to pH 7.2 and osmolarity 304 mOsmol). Spontaneous action potential-evoked postsynaptic currents (sEPSC) were recorded in ACSF without additional drugs at a holding potential of −60 mV. Intrinsic properties were recorded in current clamp mode using pipettes filled with a potassium-based solution containing (in mM) 130 K-Gluconate, 5 KCl, 10 HEPES, 2.5 MgCl2, 4 Na2-ATP, 0.4 Na3-ATP, 10 Na-phosphocreatine, 0.6 EGTA (pH 7.2 and 290 mOsmol). Resting membrane potential (vrMP), was determined directly after reaching whole-cell configuration, and the other intrinsic properties were determined with a holding potential of −60mV. Capacitance and membrane resistance were measured using a −10mV voltage step. Rheobase, threshold, maximum action potential amplitude and inter spike interval (ISI) were measured using stepwise current injection ranging from −30pA to +50pA. ISI was determined by calculating the time interval between the first and second action potential.

All recordings were acquired using a Digidata 1140A digitizer and a Multiclamp 700B amplifier (Molecular Devices, Wokingham, United Kingdom), with a sampling rate set at 20 kHz and a lowpass 1kHz filter during recording. Recordings were not analyzed if series resistance was above 25MΩ or when the recording reached below a 10:0 ratio of membrane resistance to series resistance. SEPCs were analyzed using MiniAnalysis (Synaptosoft Inc). Intrinsic properties were analyzed with Clampfit 10.7 (molecular devices, CA, USA).

**Micro-electrode Array Recordings**

Recordings of the spontaneous activity of neuronal networks derived from seven iPS lines (CTR, LH1-3, HH1-3) were performed at DIV 28. All recordings were performed using the 24-well MEA system (Multichannel Systems, MCS GmbH, Reutlingen, Germany). MEA devices allow for non-invasive recording of neuronal activity, simultaneously at multiple nodes of the same network. Each of the 24 independent wells is embedded with 12 micro-electrodes (i.e., 12 electrodes/well, 30 μm in diameter and spaced 300 μm apart). The neuronal networks could acclimatize to the recording environment (37°C; 5% CO2, 20% O2, 75% N2) for 10 minutes, after which the spontaneous neuronal network activity was recorded for a subsequent 10 minutes. The signal was sampled at 10 KHz, filtered with a high-pass filter (i.e., Butterworth, 100 Hz cut-off frequency) and the noise threshold was set at ± 4.5 standard deviations.

**Mitochondrial Morphology**

Analysis of mitochondrial morphology was done on transfected iNeurons using the DNA-In Neuro Transfection Reagent (Globalstem Inc), in combination with a DsRed2-Mito7 plasmid, which was a gift from Michael Davidson (Addgene plasmid # 55838). Normal differentiation protocol was followed as previously described, up to DIV6, where 100% of the medium was replaced with Neurobasal medium supplemented with 20 μg/mL B-27, 10 μg/mL glutaMAX, 10 ng/mL human recombinant NT3, 10 ng/mL human recombinant BDNF. A mix was made containing 0.5 μg DNA, 3 μl DNA-In Neuro reagents, and 22 μl Neurobasal medium, which was incubated at room temperature for 15 minutes, before being added to the wells in question. After 24-hour incubation, the medium is completely removed, and replaced with normal Neurobasal medium supplemented with 20 μg/mL B-27, 10 μg/mL glutaMAX, 1% Pen/Strep, 4 μg/mL Doxycycline, 10 ng/mL human recombinant NT3, 10 ng/mL human recombinant BDNF, after which the normal differentiation
The protocol is followed until DIV23. Subsequently, the cells were fixated as previously described, and an immunohistochemistry staining for MAP2 was done (see STAR Methods). Cells were imaged using a Zeiss Axio Imager Z1 equipped with apotome, using the same settings for all batches and groups at 40X magnification. Mitochondrial morphology was analyzed using a specific protocol by Valente et al. (2017), making use of the ImageJ software (Schneider et al., 2012).

QUANTIFICATION AND STATISTICAL ANALYSIS

MEA Data Analysis

MEA data analysis was performed offline by using Multiwell Analyzer (i.e., software from the 24-well MEA system that allows the extraction of the spike trains) and a custom software package named SPYCODE developed in MATLAB (The Mathworks, Natick, MA, USA) that allows the extraction of parameters describing the network activity (Bologna et al., 2010). As previously described in detail (Frega et al., 2020), the mean firing rate (MFR) of the network was obtained by computing the firing rate of each channel averaged among all the active electrodes of the MEA.

Burst detection

Burst were at least 4 spikes in burst with a minimal inter-spike-interval of 100 ms. Spike- and burst detection provided the number of active channels (spike frequency > 0.1 spikes/sec and burst rate > 0.4 burst/minute). The mean firing rate (MFR; spikes per second), the burst frequency (MBR, mean burst rate per second), the burst duration (BD, in milliseconds), the inter burst interval (IBI, in milliseconds), the mean spike frequency intra burst (MFB; spikes within a burst), and the percentage of spike outside bursts (PRS; percentage of random spikes) were obtained by computing these parameters of each separate channel and averaging these among the active electrodes of the MEA well.

Network burst detection

Synchronous events were detected looking for sequences of closely spaced single-channels bursts. A network burst was identified if it involves at least 80% of the network active channels (i.e., firing rate higher that 0.1 Hz). Furthermore, the distributions of the network burst duration (NBD, in seconds) and network inter burst interval (NIBI; the interval between network bursts, in seconds) are computed.

Network burst irregularity

Irregularity was estimated by computing the coefficient of variance (CV) of the network inter burst intervals (NIBI), as the standard deviation divided by the mean NIBI. Only networks of similar density were included for recordings. Furthermore, we always plated CTR or LH iNeurons on each MEA when HH iNeurons were plated, to ensure the general health of the MEA. Finally, when these CTR or LH iNeurons showed networks with MFR < 0.1 Hz, an absence of network bursts, or a low connectivity (< 80%) we excluded the entire MEA.

Neuronal Reconstructions

Transfected iNeurons were imaged using a Zeiss Axio Imager Z1 and digitally reconstructed using Neurolucida 360 software (Version 11, MBF–Bioscience, Williston, ND, USA). For large cells multiple 2-dimensional images of these iNeurons were taken and subsequently stitched together using the stitching plugin of FIJI 2017 software. The 3-dimensional reconstructions and quantitative morphometrical analyses focused on the somatodendritic organization of the iNeurons. We defined origins for individual primary dendrites by identifying emerging neurites with diameters that were less than 50% of the diameter of the soma. Axons, which were excluded from reconstructions and further analysis, were visually identified by their long, thin properties, far reaching projections and numerous directional changes. iNeurons that had at least two primary dendrites and reached at least the second branching order were considered for analysis. For morphometrical analysis we determined soma size, number of primary dendrites, length and branching points per primary dendrite, and total dendritic length. To measure the total span of the dendritic field (receptive area) of a neuron we performed convex hull 3D analysis. Note, that due to the 2-dimensional nature of the imaging data, we collapsed the convex hull 3D data to 2-dimensions, resulting in a measurement of the receptive area and not the volume of the span of the dendritic field. Furthermore, we performed Sholl analysis to investigate dendritic complexity in dependence from distance to soma. For each distance interval (10 μm each) the number of intersections (the number of dendrites that cross each concentric circle), number of nodes and total dendritic length was measured.

Statistical Analysis

The data are expressed as the mean ± standard error of the mean (SEM). Analysis was done using unpaired t tests, one-way analysis of variance with Bonferroni post hoc correction, or one-way repeated-measures ANOVA with sequential post hoc Bonferroni corrections, or Kolmogorov-Smirnov test, where appropriate, using GraphPad Prism 6 (GraphPad Software). P values of p < 0.05 and smaller, were deemed significant. Sample sizes were based on our previous experiences in the calculation of experimental variability, and are described, per experiment, in the corresponding figure legends. The output of all analyses is grouped per figure and combined in Tables S2–S8.