



# Revealing the Impact of Mitochondrial Fitness During Early Neural Development Using Human Brain Organoids

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Mitochondrial homeostasis -including function, morphology, and inter-organelle communication- provides guidance to the intrinsic developmental programs of corticogenesis, while also being responsive to environmental and intercellular signals. Two- and three-dimensional platforms have become useful tools to interrogate the capacity of cells to generate neuronal and glia progeny in a background of metabolic dysregulation, but the mechanistic underpinnings underlying the role of mitochondria during human neurogenesis remain unexplored. Here we provide a concise overview of cortical development and the use of pluripotent stem cell models that have contributed to our understanding of mitochondrial and metabolic regulation of early human brain development. We finally discuss the effects of mitochondrial fitness dysregulation seen under stress conditions such as metabolic dysregulation, absence of developmental apoptosis, and hypoxia; and the avenues of research that can be explored with the use of brain organoids.

**Keywords:** stem cells, glycolysis, oxidative phosphorylation, mitochondria, neural precursor cells, neural rosettes, brain organoids

## INTRODUCTION

*From the brain, and from the brain only, arise our pleasures, joys, laughter and jests, as well as our sorrows, pains, griefs, and tears. Through it, in particular, we think, see, hear, and distinguish the ugly from the beautiful, the bad from the good, the pleasant from the unpleasant, in some cases using custom as a test, in others perceiving them from their utility.*

-Hippocrates (c. 460 B.C. - c. 370 B.C.) (LOEB Classical Library, 2021)

Studies on human brain function and development have been a topic of controversy and ethical concerns throughout scientific history (Di Pietro et al., 2012; Bredenoord et al., 2017; Farahany et al., 2018; National Academies of Sciences Engineering and Medicine, 2021). It is undeniable that access to human-derived material has contributed greatly to the advance of therapies and drugs and expanded our understanding of the processes that have been studied in other biological models. The comparison between different organisms is critical to assess the specie-specific differences that have

arisen from evolution and the similarities that have been conserved and can be advantageous for the use of non-human models in translational science.

The human brain has unique characteristics that separate *Homo sapiens* from even the closest primate relatives. Cortical expansion, upper neuronal layer enlargement, increased neuronal diversity and function, complex connectivity and circuitry, are unique features of the human brain (Fuster, 2002; Kaas, 2008; Forbes and Grafman, 2010; Molnár et al., 2019; Dehay and Kennedy, 2020; Heide et al., 2020). The study of how these differences evolved has proven challenging as access to developing human tissue has been limited due to ethical considerations (Presidential Commission for the Study of Bioethical, 2015; Greely et al., 2016; Farahany et al., 2018). Additionally, the understanding of the molecular basis of various human neurological disorders has been hindered by the complexity of genetic alterations (Geschwind and Flint, 2015).

Although simple in comparison to the human central nervous system (CNS), non-human models have shown to reproduce the developmental stages, cellular composition, cytoarchitecture, and activity seen in the human brain (Arlotta and Paşca, 2019). The advent of human cellular models, like pluripotent stem cell (PSCs)-derived systems, and the intersection with research in non-human models have propelled human brain development research.

Mitochondrial diseases, which are traditionally linked to disruption in OXPHOS, are usually associated with neurological phenotypes such as developmental delay, atrophy, and epileptic encephalopathy (Ortiz-González, 2021; Povea-Cabello et al., 2021). Due to advances in exome sequencing, the range of causal mutations for mitochondrial diseases has expanded to include not only metabolic genes but proteins that affect mitochondrial shape, cristae stability, recycling, motility, and interactions with other organelles (Baum and Gama, 2021). Thus, the capacity of the mitochondria to adapt and maintain its homeostasis its key for the correct execution of the intrinsic developmental programs of neural and corticogenesis, while also being responsive to environmental and intercellular signals.

Remodeling of the mitochondrial network as human pluripotent stem cells (hPSCs) commit to a neuronal cell fate is necessary for their survival and function (Chan, 2012; Schwarz, 2013; Khacho et al., 2016; Khacho and Slack, 2018; Iwata et al., 2020). Likewise, neural processes such as development, migration, maturation, and plasticity; demand high levels of energy (Klein Gunnewiek et al., 2020). Mitochondrial fragmentation is a hallmark of glycolytic cell types such as stem cells (Teslaa and Teitell, 2015; Chen and Chan, 2017; Zhang H. et al., 2018; Rastogi et al., 2019), and the ability to transition to a more complex and elongated network that facilitates aerobic respiration is crucial for the survival of the newborn neurons (Chan, 2012; Schwarz, 2013; Zheng et al., 2016).

in the first part of this review we provide an overview of cortical development and the use of PSC models. In the second part, we discuss our current understanding of mitochondrial and metabolic regulation during early human brain development and the effects of mitochondrial fitness dysregulation seen under stress conditions such as metabolic dysregulation, absence of developmental apoptosis, and hypoxia.

## Human Brain Development: Finely Orchestrated Events of Commitment, Migration, and Expansion

The human CNS is composed of approximately 86 billion neurons, with a roughly equal number of glial cells (Herculano-Houzel et al., 2015). Ninety-nine percent of all neurons are located inside the cranium. The cerebral cortex is composed by ~20% of all neurons, although it represents ~50% of the CNS volume (Molnár and Pollen, 2014; Herculano-Houzel et al., 2015, 2016; Sousa et al., 2017).

The human brain has specie-specific characteristics that highlight the need for more representative modeling of development. For example, the developing brain has expanded proliferative zones (e.g., outer subventricular zone, SVZ) with diverse subtypes of neural stem and progenitor cells [e.g., outer radial glia (oRGCs)] that facilitate the expansion of the neocortex (Bystron et al., 2008; Sousa et al., 2017; Molnár et al., 2019). The timing and duration of neurogenesis is also a factor to consider when examining the differences between species. Extended human neurogenesis results in an increased number of progenitor cells that give rise to larger neocortical structures with increased number of upper-layer neurons (Smart et al., 2002; Hutsler et al., 2005; Taverna et al., 2014). In addition to the ventricular zone (VZ) and the cortical plate (discussed in detail below), the human brain contains an additional layer, called the outer SVZ. The outer SVZ is characteristic for primate brains and it contains oRGCs or basal radial glia cells. In rodent brains, these cells are not present, or present in only very small numbers (Wang L. et al., 2011; Wang X. et al., 2011; Kalebic and Huttner, 2020). But, in primates, they act as a transit amplifying population during neurogenesis (Fietz et al., 2010; Hansen et al., 2010; Sauerland et al., 2018) contributing to the expansion of the cortex. Developmental studies in rodents segregate the birth of excitatory neurons and interneurons to the progenitors in the cortex and ganglionic eminence, respectively, yet lineage tracing of primary human neural progenitors show that individual cortical progenitors have the capacity to generate both excitatory and cortical interneurons (Delgado et al., 2022).

Human brain development is a prolonged and intricate process that starts around 2 weeks post conception and continues until early adulthood. The initial stages of this process rely primarily on the genetic control and the correct activation of neural programs, although, environmental factors can also affect the efficiency of the process.

### Initial Development: Neural Tube Formation

The genesis of the nervous system initiates about 2 weeks post conception. At this stage, the developing embryo is organized as a three-layered spherical structure. Cells in the ectoderm, one of the three germ layers, thicken to form the neural plate. The lateral edges of the neural plate will give rise to the neural fold that will join at the midline forming the neural tube. The closure of these tube occurs from the center to the cranial and caudal ends. Developmental defects at this point can cause anencephalia or spina bifida. The formation of the neural tube leads to the formation of the CNS by the cells located in the inner part of

the tube, while the outer cells will give rise to the peripheral nervous system (O'Rahilly and Müller, 2005; Bayer and Altman, 2007).

Once the neural tube closes, around 4 weeks post-conception, the cranial end will expand to become a three-vesicle structure: the prosencephalon, the mesencephalon, and the rhombencephalon. By week 5 post-conception, the prosencephalon will give rise to the telencephalon, which will correspond with the forebrain and includes the cerebral hemispheres; and the diencephalon that will develop into the thalamus and hypothalamus (O'Rahilly and Müller, 2008). The mesencephalon will give rise to the mature midbrain; and the rhombencephalon will generate the metencephalon which in time will derive the pons and the cerebellum, and the myelencephalon that will develop into the medulla (O'Rahilly and Müller, 2005, 2010; Bayer and Altman, 2007).

Morphogen signaling during this period is crucial for the establishment of the development axis. The notochord, a structure derived from the axial mesoderm immediately ventral to the ectoderm (Grow, 2018; Di Gregorio, 2020), and the somites, transient paired structures derived from mesenchymal paraxial mesoderm that flank the neural tube (Cook et al., 2017), define the dorso-ventral axis of the embryo by releasing different signaling molecules (Seal and Monsoro-Burq, 2020).

#### **Fibroblast Growth Factor**

FGF8 is produced by the paraxial mesoderm and it's downregulated before neural differentiation (Bertrand et al., 2000; Muñoz-Sanjuán and Brivanlou, 2002). This downregulation is necessary for the expression of early neural transcription factors such as NEUROM, PAX3, HES4, TFAP2A, and MSX1 in the neural tube (Bang et al., 1997; Diez del Corral et al., 2002; Monsoro-Burq et al., 2005; Garnett et al., 2012).

#### **Retinoic Acid**

Retinoic acid (RA) signaling is generated by the paraxial mesoderm for the induction of neural differentiation and patterning by downregulating fibroblast growth factor (FGF) production (Franco et al., 1999; Colas and Schoenwolf, 2001; Diez del Corral and Storey, 2004). RA generated in the SVZ of the basal ganglia is required for GABAergic differentiation (Chatzi et al., 2011), whereas the RA generated in the meninges regulates cortical neuron generation (Siegenthaler et al., 2009). Moreover, classic studies in *Xenopus* implicated WNT (blend of the names Wingless and Int-1), RA and FGF as dorsalizing factors (Ruiz I Altaba and Jessell, 1991; Sharpe, 1991; Altmann and Brivanlou, 2001).

#### **Sonic Hedgehog**

Sonic hedgehog (SHH) is produced by the notochord for the ventralization of neural cell types in a concentration dependent manner (Jessell and Dodd, 1990; Echelard et al., 1993; Liem et al., 2000). Mouse embryos lacking SHH fail to form ventral telencephalons and show a marked reduction on the expression of ventral markers (Rallu et al., 2002), and its ectopic expression is sufficient to induce the expression of ventral telencephalic *in vitro* and *in vivo* (Gaiano et al., 1999).

#### **WNT**

The activation of WNT signaling, especially from Wnt3a, is necessary for the induction of posterior patterning (McGrew et al., 1995; Kiecker and Niehrs, 2001). WNT signaling repression is critical for the generation of the anterior neural fate (Yamaguchi, 2001).

#### **Bone Morphogenetic Protein**

Bone morphogenetic protein (BMP) are produced by the non-neural ectoderm and the ventral mesoderm. High BMP signaling in the ventral ectoderm promotes the epidermal fate and represses the neural fate. BMP antagonists, such as Noggin, Chordin, Cerberus and Follistatin, are secreted by the Spemann-Mangold organizer (Spemann and Mangold, 1924, 2001; Muñoz-Sanjuán and Brivanlou, 2002), generating a low-to-high gradient of BMP signaling from the midline toward the lateral zones that allows for the neural specification of the dorsal ectoderm (Lee et al., 1998; Barth et al., 1999; Lee and Jessell, 1999; Liem et al., 2000; Nguyen et al., 2000; Ybot-Gonzalez et al., 2007).

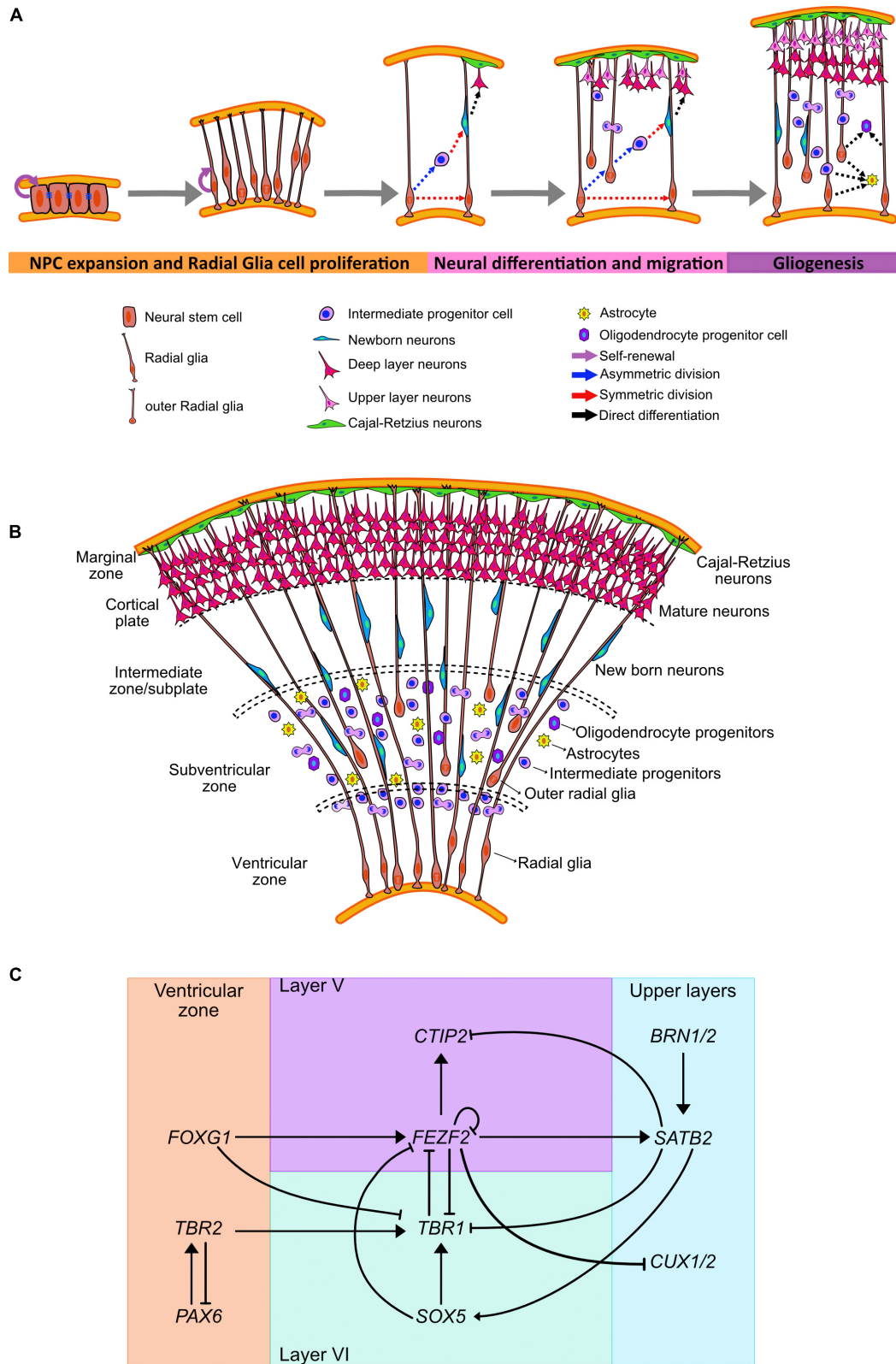
#### **Neural Progenitor Cell Expansion and Radial Glia Cell Proliferation**

Once the closure of the neural tube is complete, the cells lining the lumen of the tube will develop into the ventricles. This single cell layer of neuroepithelia is a pseudostratified epithelium composed by neuroepithelial progenitor cells (Götz and Huttner, 2005; **Figure 1A**). These are highly polarized along the apical-basal axis (Chenn et al., 1998). Transmembrane proteins such as prominin-1 are found in the apical plasma membrane, while tight and adherens junctions are present at the apical end of the lateral plasma membrane (Aaku-Saraste et al., 1996; Chenn et al., 1998; Zhadanov et al., 1999; Manabe et al., 2002). Receptors for basal lamina components, such as integrins, are located in the basal plasma membrane that is in contact with the basal lamina (Wodarz and Huttner, 2003).

This epithelium appears as stratified due to the cell nuclei migrating up and down the apical-basal axis during the cell cycle in a process known as interkinetic nuclear migration (His, 1889; Schaper, 1897; Sauer, 1935; Frade, 2002; Bayer and Altman, 2007; Rodrigues et al., 2019). In this process the nuclei migrate to the apical side during mitosis and remains basally during the S phase. This movement exposes the nuclei to diffused morphogens, such as Notch receptor ligand Delta, and influence the fate of the daughter cells (Chenn and McConnell, 1995; Chenn et al., 1998; Murciano et al., 2002; Del Bene et al., 2008; Spear and Erickson, 2012; Azizi et al., 2020).

The neuroepithelial progenitor cells divide in either a symmetric or asymmetric manner (Huttner and Brand, 1997; Götz and Huttner, 2005). Symmetric cell division generate two daughter cells that remain mitotically active and can expand the population of progenitor cells (Kornack and Rakic, 1995; Mione et al., 1997). In contrast, in an asymmetric division at least one of the daughter cells exit the cell cycle and differentiates into a specialized cell (Reid et al., 1997; Qian et al., 1998; Fishell and Kriegstein, 2003; Wodarz and Huttner, 2003).

Rounds of cell division from the neuroepithelial cells form several layers surrounding the lumen (**Figures 1A,B**). The



**FIGURE 1 |** Human neocortical development. **(A)** Schematic illustration of neurogenesis in the human cortex. **(B)** Cortical expansion in humans allows for the formation of different areas where progenitor lineages migrate, proliferate, and differentiate. **(C)** Transcriptional regulators and genes governing cell fate acquisition and specification during neurogenesis.

inner-most apical layer becomes populated with the progenitor cells and due to the proximity with the ventricles is known as the VZ. As asymmetric division mark the beginning of neurogenesis, neuroepithelial cells have been shown to downregulate the expression of tight junction and certain apical plasma membrane proteins (Aaku-Saraste et al., 1996, 1997).

Neuroepithelial cells give rise to a progenitor cell type that is more cell fate-restricted, the radial glial cells (RGCs) (Magini, 1888; Koelliker, 1896; Bentivoglio and Mazzarello, 1999). These cells can be observed in the developing embryo as early as week 6 post conception (Choi, 1981). RGCs are characterized by bipolar processes extending to reach the pial and ventricular surfaces, while their cell bodies remain in the VZ (Rakic, 1972, 1978; Levitt and Rakic, 1980; Haubensak et al., 2004). These processes help guide the radial migration of newborn neurons from the VZ. Moreover, these cells share some astrocytic characteristics such as glycogen granules and the expression of the intermediate filament protein, glial fibrillary acidic protein (GFAP) (Levitt and Rakic, 1980; Choi, 1981) and VIMENTIN, as well as brain-lipid-binding protein (BLBP) (Hartfuss et al., 2001). RGCs also maintain the expression of the neuroepithelial marker NESTIN (Pixley and de Vellis, 1984; Chanas-Sacre et al., 2000; Campbell and Götz, 2002), apical-basal polarity with the presence of adherence junctions (Aaku-Saraste et al., 1996; Wodarz and Huttner, 2003), basal lamina contact (Halfter et al., 2002), and interkinetic nuclear migration. Regionalization of the brain is also an ongoing process at this stage. Dorsal RGCs express the neural progenitor marker Paired box protein 6 (PAX6) (Götz et al., 1998) which commit them into a cortical fate; while the ventral RGCs express the cellular retinol-binding protein (RBP-1) (Toresson et al., 1999) and will commit to form the basal ganglia.

Prior to the peak in neurogenesis, RGCs divide symmetrically to amplify the progenitor cell population (**Figure 1A**). However, during the peak phase of neurogenesis, RGCs primarily divide asymmetrically to both self-renew and give rise to outer RGCs (oRGCs), intermediate progenitors (IPCs), astrocytes, or neurons (Malatesta et al., 2000, 2003; Alvarez-Buylla et al., 2001; Miyata et al., 2001; Noctor et al., 2001; Anthony et al., 2004; Kriegstein and Alvarez-Buylla, 2009). The cell fate specification during the asymmetrical neurogenesis has been associated with the subcellular distribution of the mammalian partition defective protein 3 (mPAR3) by differentially regulating Notch signaling activity in the two daughter cells (Bultje et al., 2009).

Outer radial glia and intermediate progenitors will establish the SVZ around week 9 post conception (Zecevic et al., 2005). oRGs retain the basal processes but lack the apical junctions (Miyata et al., 2001), and undergo a distinct migratory behavior or mitotic somal translocation before undergoing cell division (Fietz et al., 2010; Hansen et al., 2010; Wang X. et al., 2011). These cells can also undergo proliferative and asymmetric cell divisions and require Notch signaling to induce neuronal differentiation (Kowalczyk et al., 2009; Hansen et al., 2010). IPCs are transient amplifying cells with limited proliferative divisions, which are predominantly symmetrical to produce two neurons (Noctor et al., 2001; Haubensak et al., 2004; Wu et al., 2005; Sessa et al., 2008, 2010; Kowalczyk et al., 2009). These cells have multipolar morphology and are not anchored to either the apical or basal

cortical surface (Noctor et al., 2001; Haubensak et al., 2004). IPCs contribute to radial expansion and folding of the human brain (Englund, 2005; Baala et al., 2007; Miller et al., 2019) and have been associated to the generation of the upper cortical layers (Martínez-Cerdeño et al., 2006; Arnold et al., 2008; Lv et al., 2019).

The different types of progenitors can be identified not only by their morphology and presence of polarizing membrane proteins, but by the expression of certain fate markers (**Figure 1C**). Radial glia express PAX6, a homeodomain transcription factor, in contrast IPCs upregulate T-Box Brain Protein 2 (TBR2), a T-domain transcription factor, and downregulate PAX6 (Englund, 2005). oRGCs and SVZ progenitors also express the non-coding RNA subventricular-expressed transcript 1 (SVET1) (Tarabykin et al., 2001), as well as the transcription factor Cut Like Homeobox2 (CUX2) (Nieto et al., 2004; Zimmer et al., 2004). Moreover, oRGs preferentially express genes related to extracellular matrix formation, migration, and stemness (Pollen et al., 2015; Nowakowski et al., 2017; Fan et al., 2020).

### Corticogenesis: Neural Differentiation and Migration

The generation of cortical neurons, or corticogenesis, can be distinguished by the enlargement of the SVZ that has an inner (ISVZ) and outer region (OSVZ), and it is separated by a thin fiber layer (Zecevic et al., 2005). Cells undergoing mitosis can be observed in all levels of the SVZ, in contrast with the VZ where mitotic cells are only found in the ventricular surface (Smart et al., 2002; **Figures 1A,B**).

Early born neurons migrate away from the ventricular surface, segregating themselves from the progenitors and forming the pre-plate. The first wave of neurons is composed by specialized pioneering cells or Cajal-Retzius neurons. These neurons organize adjacent to the pial surface forming the marginal zone, that in the adult human brain correspond with Layer I (Marin-Padilla, 1978; Raedler and Raedler, 1978) and that act as the stop sign for neuronal migration (Tissir and Goffinet, 2003). These cells secrete REELIN, a large extracellular matrix glycoprotein. REELIN regulate processes of neuronal migration and positioning in the developing brain by controlling cell-cell interactions specifically by binding to the transmembrane receptors very low-density lipoprotein receptor (Vldlr), and apolipoprotein E receptor 2 (ApoER2) present on migrating neurons (D'Arcangelo et al., 1995; Hiesberger et al., 1999). The binding of REELIN to the previously mentioned receptors induces the phosphorylation of Disabled 1 (Dab1), a cytosolic protein that activates tyrosine kinases (Trommsdorff et al., 1999; Kim et al., 2002) that in time modulates the phosphorylation of Tau affecting the assembly and stability of the neuronal cytoskeleton (Hiesberger et al., 1999; Rice and Curran, 2001). REELIN also interacts with  $\alpha\beta 1$  integrin, which regulates neuron-glia interactions by promoting the detachment of the migratory neuron from the radial glia via Dab1 and is required to achieve proper laminar organization (Dulabon et al., 2000).

As new neurons are born and start migrating into the pre-plate, this area is divided into the marginal zone, which is pushed outwards, and the subplate (Molnár et al., 2019). The subplate is a voluminous yet transient compartment in the cerebral wall

composed of post-migratory and migratory neurons, glia and axons; as well as of abundant extracellular matrix (Kostovic and Rakic, 1990; Hoerder-Suabedissen and Molnár, 2015). This structure is key for the correct formation and subsequent function of the brain as it is involved in the formation of neural circuits (Kostovic and Rakic, 1990).

Between the marginal zone and the subplate, newborn neurons migrate and organize forming the cortical plate around post-conception weeks 8–9 (Clancy et al., 2001). In this area, the nascent neurons stop migrating and differentiate into their final laminar identity in an inside-out fashion (Greig et al., 2013), with newer born neurons positioning closer to the marginal zone (Molyneaux et al., 2007; Leone et al., 2008; Marín-Padilla, 2014; Shibata et al., 2015). Hence, neurons of the deepest layers (VI and V) are generated at the earliest stages, followed by neurons of layers IV, III, and II. For the neuronal maturation process, final positioning of the neurons is required; and can last until early adulthood in humans (Huttenlocher, 1979; Petanjek et al., 2008, 2011).

Cortical neurons are generated primarily (~80%) by IPCs, while the other 10–20% can be traced back to RGCs (Kowalczyk et al., 2009; Vasistha et al., 2015). Moreover, cortical progenitors undergo fate restriction as they produce new neurons. Late cortical progenitors are not capable to generate earlier neuronal fates even when exposed to environments that mimic the early stages of corticogenesis (Walsh and Cepko, 1993; Frantz and McConnell, 1996; Desai and McConnell, 2000). This fate restriction has been associated with changes in the length of the cell cycle and the number of divisions before terminal differentiation (Calegari et al., 2005; Shen et al., 2006; Pilaz et al., 2009).

Fate acquisition has also been linked with the expression of subtype-specific transcription factors expressed in progenitors before neurogenesis; suggesting an early commitment to a particular cortical layer (Greig et al., 2013; **Figure 1C**). The transition from the pioneering Cajal-Retzius cells into the generation of deep-layer neurons has been shown to be mediated by the repression of the transcription factor Forkhead Box G1 (FOXP1) in neural progenitors (Hanashima et al., 2004).

Deep-layer progenitors express the transcription factors FEZF2 (Fez family zinc finger 2), OTX1 (Orthodenticle Homeobox 1), and EMX2 (Empty Spiracles Homeobox 2) (Frantz et al., 1994; Leingärtner et al., 2003; Inoue et al., 2004; Chen et al., 2005; McKenna et al., 2011; Lodato et al., 2014) that have been associated with neurons present in layers VI and V. OTX1, for example, is expressed in VZ precursors that will give rise to cortical layers VI and V, but it is downregulated in progenitor cells that are committed to upper layer neurogenesis (Frantz et al., 1994; Weimann et al., 1999).

The generation of layer VI neurons, around post-conception weeks 11–12 (Clancy et al., 2001), is mediated by the expression of the transcription factor T-Box Brain Protein 1 (TBR1) (Hevner et al., 2001; Bedogni et al., 2010). TBR1 downregulates the expression of FEZF2 (Han et al., 2011) and CTIP2 (COUP-TF-Interacting Protein 2) (McKenna et al., 2011) by binding to the FEZF2 gene and inhibiting its transcription. FEZF2 acts upstream of CTIP2 to control the differentiation of layer V neurons (Arlotta et al., 2005; Chen et al., 2005) by reducing TBR1 expression

(McKenna et al., 2011). TBR1 expression is also downregulated directly by FOXP1 and indirectly by the FOXP1 mediated upregulation of FEZF2 (Toma et al., 2014). Moreover, SOX5 (Sex Determining Region Y-Box 5) directly represses FEZF2 by binding to a required enhancer element for its expression (Kwan et al., 2008; Lai et al., 2008; Shim et al., 2012) and induces the activation and maintenance of TBR1 (Bedogni et al., 2010).

As deep layer progenitors express OTX1 before neurogenesis, upper layer progenitors express the non-coding RNA SVET1. The expression of this marker is exclusive of layer II–IV and its activation is dependent on the correct expression of PAX6 (Tarabykin et al., 2001). Expression of SVET1 has been also identified in IPC-derived upper cortical layer neurons (Molyneaux et al., 2007).

Transition to the production of upper layer neurons is mediated by the interaction of FEZF2, CTIP2, TBR1, and SATB2 (Special AT-Rich Sequence-Binding Protein 2). TBR1 is a downstream target of SATB2, and its repression is necessary for the differentiation of layer IV neurons (Srinivasan et al., 2012). Moreover, downregulation of FEZF2 by negative feedback (Toma et al., 2014) is necessary for the acquisition of SATB2 expression (Chen et al., 2008), and in time, SATB2 binds to and represses the expression of CTIP2 (Alcamo et al., 2008; Britanova et al., 2008).

Co-expression of Brain-Specific Homeobox/POU Domain Protein 1 (BRN1) and Brain-Specific Homeobox/POU Domain Protein 2 (BRN2/POU3F2) in most layer II–V cortical neurons is preceded by its expression in VZ progenitors and they are required for the control of radial migration in neurons residing in those cortical layers (McEvelly et al., 2002; Dominguez et al., 2013). Moreover, disruption in the expression of these transcription factors causes defective migration of neurons due to the inability of the neurons to express Dab1 (Sugitani et al., 2002).

Expression of the transcription factor CUX2 in progenitors has been associated with the generation of cortical neurons from layers II/III (Nieto et al., 2004; Zimmer et al., 2004; Molyneaux et al., 2009) at post-conception week 12 (Clancy et al., 2001). This predisposition to generate upper layer neurons is observed in the progenitor cells in the VZ that undergo proliferative cell division during the phase of deep-layer neuronal generation. These cells then switch to neurogenic differentiation to generate superficial-layer neurons (Franco et al., 2012). CUX1 and CUX2 are also part of the regulatory network modulated by FEZF2, where its inactivation allows for the progression to upper layer specification (Lodato et al., 2014).

## Gliogenesis

During cortical neurogenesis, the promoters for astrocytic fate, such as GFAP, are heavily methylated and inaccessible to be activated by STAT3 (Signal Transducer And Activator Of Transcription 3) (Takizawa et al., 2001). Expression of Neurogin 1 (NGN1) competes with STAT3 for the EP300-SMAD activator complexes and suppress the JAK/STAT and BMP signaling pathway (Sun et al., 2001). At the end of neurogenesis, levels of NGN1 drop and the GFAP promoter is demethylated to induce astrocytic fate and promote the differentiation of radial glia cells into glial progenitors (Takizawa et al., 2001). This demethylation of the astrocyte-specific genes is mediated by the Notch signaling pathway activation of the nuclear factor IA

(NFIA) (Deneen et al., 2006; Namihira et al., 2009). NFIA is also directly regulated by SOX9 (Sex Determining Region Y-Box 9), with the SOX9/NFIA complex directly regulating genes implicated in astrocyte precursor migration and metabolism (Kang et al., 2012).

Astrocytes arise from radial glia in the VZ and PAX6+/HOPX+ oRGs in the outer SVZ (Cameron and Rakic, 1991; Noctor et al., 2008; Kriegstein and Alvarez-Buylla, 2009; Ge et al., 2012; Zweifel et al., 2018; Rash et al., 2019) around post-conception week 12 (Empie et al., 2015). Local proliferation of differentiated astrocytes that undergo symmetric division generate mature astrocytes (García-Marqués and López-Mascaraque, 2013) that are able to form endfeet with blood vessels and are perform functions such as glutamate uptake (Ge et al., 2012).

Another class of glial cells, oligodendrocytes, originate from precursors in the proliferative dorsal and ventral zones of the developing brain (Richardson et al., 2006). These cells migrate to the developing white matter, divide, and terminally differentiate. The activation of the transcription factors OLIG1 (Oligodendrocyte Transcription Factor 1) and OLIG2 (Oligodendrocyte Transcription Factor 2) are indispensable for the oligodendroglial fate acquisition. OLIG1 directly binds to DLX1/2 (Distal-Less Homeobox 1/2) enhancers to repress neurogenic fate and induce the expression of OLIG2 (Petryniak et al., 2007; Silbereis et al., 2014). ASCL1 (Achaete-Scute Family BHLH Transcription Factor 1) is also downregulated in the transition from neural progenitor cells (NPCs) to oligodendrocyte progenitor cell, although its expression in these cells is biphasic as it needs to be upregulated again for oligodendrocyte terminal differentiation (Battiste et al., 2007; Dimou et al., 2008; Sugimori et al., 2008). Expression of several SOX (Sex Determining Region Y-Box) members is key for the specification and differentiation of oligodendrocytes. SOX5 and SOX6 are expressed in oligodendrocyte progenitor cells and influence migration patterns, while SOX10 induces terminal differentiation and myelin gene expression (Stolt et al., 2002, 2006).

Sonic Hedgehog signaling, through the activation of OLIG2, is necessary for oligodendrocyte development (Agius et al., 2004); yet their maturation is SHH independent (Orentas et al., 1999; Soula et al., 2001). Expression of the EGF receptor has also been associated with the generation of OPCs (Huang et al., 2020). EGFR-expressing oRGs may act as intermediate progenitors for oligodendrogenesis and potentially amplify the OPC progenitor pool (Huang et al., 2020). BMP and WNT expression have been shown to oppose oligodendrocyte cell fate and directing the progenitors into an astrocyte fate (Mekki-Dauriac et al., 2002; Samanta and Kessler, 2004; Shimizu et al., 2005).

## HUMAN PLURIPOTENT STEM CELL DERIVED MODELS FOR THE STUDY OF BRAIN DEVELOPMENT

Animal models (Jain et al., 2016, 2019; Ferrari et al., 2017) and brain tissue from biopsies have provided critical insight into mitochondrial disease. However, our understanding of

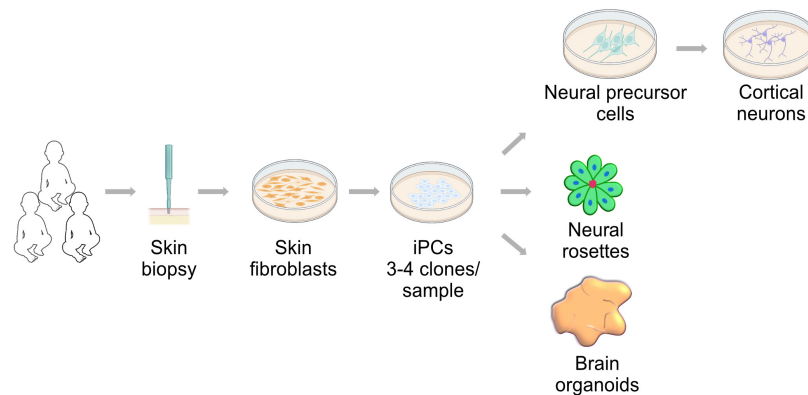
the etiology and pathology of complex mitochondrial diseases would benefit from the use of human-derived platforms such as induced PSC-derived models described in this section. Human induced pluripotent stem cells (iPSCs) have been generated from patients with mutations in mitochondrially encoded ATP Synthase Membrane Subunit 6 (MT-ATP6) (Ma et al., 2015; Galera-Monge et al., 2016; Lorenz et al., 2017; Grace et al., 2019), mitochondrially encoded NADH:Ubiquinone Oxidoreductase Core Subunit 3 (MT-ND3) subunit (Hattori et al., 2016), and the nuclear-encoded gene Surfeit locus protein 1 (SURF1) (Inak et al., 2021). These iPSC-model systems are useful tools for drug discovery (Inak et al., 2017; Lorenz et al., 2017) as well as testing platforms for potential metabolic rescue treatments (Ma et al., 2015).

The study of human embryonic development described in the previous section, specifically organogenesis and cell fate specification, has been greatly improved by the isolation and maintenance of PSCs (Evans and Kaufman, 1981; Thomson et al., 1995, 1998; Thomson and Marshall, 1998). The access to somatic cells and the ability to reprogram them into induced PSCs (Takahashi and Yamanaka, 2006; Takahashi et al., 2007) has enabled modeling developmental diseases that are considered rare or difficult to phenocopy in model organisms, including mitochondrial diseases (Saha and Jaenisch, 2009). This, coupled with the ability to generate differentiated cells such as neurons (Zhang et al., 2001; Vierbuchen et al., 2010), has expanded the technical approaches available to study human brain development in vitro (**Figure 2**). Here we provide an overview of current iPSC-derived systems and the developmental insight we can gain from their use. These models could expand the repertoire available to understand the contribution of mitochondrial biology and function to human brain development and could expand our view of mitochondrial diseases.

## Human Pluripotent Stem Cell-Derived Neurons

The generation of neural cells was based on observations of the grafting effects of mouse teratocarcinoma lines in early embryos and in vitro (Jones-Villeneuve et al., 1983). Cell aggregation in addition to treatment with RA promoted the differentiation of mouse embryonic stem cells (mESCs) into cells expressing neuronal genes and capable of generating action potentials (Bain et al., 1995). The use of morphogens for the maintenance and generation of neuroepithelial precursors was highlighted by the use of basic fibroblast growth factor (bFGF or FGF2) (Okabe et al., 1996; Mujtaba et al., 1999). Removal of this compound promoted the differentiation of functional neuronal cells that were able to be cultured long term (Okabe et al., 1996). Furthermore, these ESCs-derived neural precursors were shown to be able to graft to embryonic ventricles and migrate without positional cues into the host brain to contribute to the three lineages of the nervous system (Brüstle et al., 1997).

The first account of human neural precursor cells differentiation from hESC was reported by the Thomson and Ben-Hur laboratories in 2001. After aggregation in embryoid bodies, neural tube-like structures were generated in the presence of FGF2 (Reubinoff et al., 2001; Zhang et al., 2001). Removal



**FIGURE 2 |** Neural development research approach utilizing induced pluripotent stem cells (iPSCs). Skin fibroblast can be derived from patients and controls by a minimally invasive biopsy. These fibroblasts can be reprogrammed using the Yamanaka factors into induced pluripotent stem cells. iPSCs can generate neural cells in two- and three-dimensional cultures. These approaches are powerful tools to study of neural development in health and mitochondrial disease conditions.

of this morphogen promoted differentiation into the neural lineages *in vitro*, while transplantation into the neonatal mouse brain allowed for their incorporation to different brain regions and further maturation into neurons and astrocytes.

An improved protocol that did not rely on embryoid bodies was generated by the Studer lab in 2009. Rapid and highly efficient neural conversion under adherent culture conditions was obtained by the synergistic use of two SMAD signaling pathway inhibitors: Noggin and SB431542 (Chambers et al., 2009). Noggin is a bone morphogenic protein (BMP) inhibitor identified initially in *Xenopus laevis* with neural-inducing properties following a dorsal fate pattern (Smith and Harland, 1992; Valenzuela et al., 1995; Gerrard et al., 2005; Lee et al., 2007). The drug SB431542 is an antagonist that inhibits the Nodal/Activin/Transforming growth factor  $\beta$  (TGF $\beta$ ) pathways by blocking phosphorylation of the ALK4, ALK5 and ALK7 receptors. Activin and Nodal are responsible of mesodermal and endodermal differentiation during gastrulation, the inhibition of these pathways allows for an ectodermal and neural fate induction both *in vivo* and *in vitro* (Schier, 2003; Smith et al., 2008).

Neural progenitor cell fate is determined by the expression of neuroectoderm markers such as Sex Determining Region Y-Box 1 (SOX1), PAX6 and Neuroepithelial stem cell protein (NESTIN). The intermediate filament NESTIN is expressed in both neuroepithelial cells and RGCs with different morphologies depending on the cell type and size (Götz and Barde, 2005). Expression of SOX1 can be observed upon neuroectoderm formation. Subsequent expression of PAX6 is noted as early as the first somite formation and the closing of the neural fold in the cranial region (E8.0 in mouse and stage 10 in human). PAX6 is also expressed in RGCs (Callaerts et al., 1997; Englund, 2005; Thakurela et al., 2016). The switch between SOX1 and PAX6 during neural fate commitment is required for the correct differentiation into committed cell types. Continuous expression of SOX1 inhibits the expression of PAX6 and other RGC markers. PAX6 expression induces cell migration and differentiation into neurons (Heins et al., 2002; Cartier et al., 2006; Suter et al., 2009).

Further differentiation to different neuronal cell types from different regions of the CNS have been achieved (Pankratz et al., 2007; Zeng et al., 2010). Previous studies using mice and other model organisms expanded the knowledge on morphogens and growth factors required for neural development (Gaspard et al., 2008). NPCs with anterior patterning fate have been used to generate dopaminergic neurons after exposure to FGF8 for anterior–posterior axis induction and signal SHH as a ventralizing signal (Perrier et al., 2004). Spinal motoneurons can be generated by the presence of SHH and the retinoic acid to promote the posterior axis fate (Li et al., 2005; Lee et al., 2007). Functional cortical glutamatergic neurons and telencephalic GABAergic neurons can also be generated by the manipulation of the endogenous WNT signaling in NPCs (Li et al., 2009). Cerebral cortex development can also be recapitulated by the sequential generation of pyramidal neurons that express the markers of the different cortical layers (Shi et al., 2012; Espuny-Camacho et al., 2013).

As an alternative to hPSC-derived neurons, direct differentiation from somatic cells into functional NPCs and neurons have been achieved, using either genetic or chemical manipulation of the embryonic pathways that promote transdifferentiation. The transcription factors BRN2, ASCL1, Myelin Transcription Factor 1-Like Protein (MYTL1), and Neurogenic differentiation 1 (NEUROD1); as well as the microRNAs miR-9\* and miR-124, were initially used to generate different types of neurons from fibroblasts (Ambasudhan et al., 2011; Caiazzo et al., 2011; Pang et al., 2011; Pfisterer et al., 2011a,b; Son et al., 2011; Yoo et al., 2011). Single transcription factors, such as Sex Determining Region Y-Box 2 (SOX2) (Ring et al., 2012) and NEUROGENIN-2 (NGN2) (Zhang et al., 2013) have been used to generate functional excitatory cortical neurons.

## Human Pluripotent Stem Cell-Derived Neural Rosettes

The earliest *in vitro* recapitulation of nervous system development are neural rosettes (NR). These structures correspond to the third week of gestation



(Chandrasekaran et al., 2017) and recapitulate the initial stages of CNS development. NRs are composed of long, radially organized, columnar neuroepithelial cells surrounding a central cavity or lumen (Zhang et al., 2001; Elkabetz et al., 2008; Chandrasekaran et al., 2017; Hříbková et al., 2018). NRs present apical-basal polarity, with the localization of apical markers (e.g., ZO1, N-Cadherin,  $\beta$ -Catenin) and interkinetic nuclear migration. Additionally, cells in NRs are positive for neuroepithelial markers such as PAX6, SOX1, NESTIN, Mushahi-1 (MSI1) and polysialylated neuronal cell adhesion molecule (PSA-NCAM). These cells also show self-renewal capacity, engraftment capacity, and can differentiate into different region-specific neural and glial types in response to developmental cues (Zhang et al., 2001; Perrier et al., 2004; Li et al., 2005; Elkabetz et al., 2008; Koch et al., 2009; Grabiec et al., 2016; Knight et al., 2018).

Neural rosettes-neural stem cells (NR-NSCs) can be isolated, expanded, and regionally specified without losing the rosette properties. Maintenance of the multipotency capacity has been shown to depend on the Notch signaling pathway, as low plating densities or the pharmacological inhibition of the pathway, increases the neuronal differentiation and causes a reduction in the rosette formation efficiency. Activation of the SHH has also been shown to be a key pathway in the maintenance of multipotency of NR-NSCs. Inhibition of both pathways cause rapid loss of rosette organization and reduction in the differentiation capacity (Elkabetz et al., 2008).

Although the default fate pathway for NR cells is the anterior CNS pattern by the expression of FOXG1 (Tao and Lai, 1992; Koch et al., 2009), re-specification toward caudal fates can be accomplished: midbrain and hindbrain neurons can be obtained by FGF8/SHH treatments, and spinal motor neurons can be generated by incubation in RA/SHH (Perrier et al., 2004; Elkabetz et al., 2008). Furthermore, NRs with ZO1 expression and interkinetic nuclear migration can also be spontaneously generated from NPC monolayer differentiation via dual SMAD inhibition (Chambers et al., 2009).

Neural rosette formation consists of five morphogenetic changes: intercalation of two or more cell rows, cellular constriction or shrinkage, polarization, elongation, and lumen formation. The formation of the lumen is mediated by apoptosis, inhibition of cell death via caspase inhibition disrupts lumen formation and delay neurogenesis.  $Ca^{2+}$  regulation is also critical in the formation of the NR by regulating multiple cytoskeletal proteins during the first three morphogenesis events. Calcium dysregulation also affects the localization of the polarizing proteins ZO1, PARD3 and  $\beta$ -catenin, which in turn impairs lumen formation. Disruption of the cytoskeleton (specifically actin, myosin II and tubulin) promotes premature neurogenesis and astrogenesis (Hříbková et al., 2018).

FGF and BMP signaling are also necessary for the correct formation of the NRs. FGF2 and its receptor FGFR1 present an apical polarization in the lumen of the NR. FGF2 overexpression or inhibition disrupts the formation of the NRs and affects ZO1 expression and localization. The malformation of the NRs can be mediated by the displacement of ZO1 from the apical membrane which in time disrupts the membrane anchorage of FGFR1, causing the disruption on FGF2 signaling gradient, reduced cell proliferation, cell cycle exit and the premature differentiation

of the NPCs (Grabiec et al., 2016). BMP signaling has been associated to the complex cluster formation of the NR. Inhibition of the pathway causes disrupted rosette morphology and alters the expression of the NSC markers PAX6, SOX2, and SOX1 (Fedorova et al., 2019).

## Human Pluripotent Stem Cell-Derived Brain Organoids

The brain organoid field started with the groundbreaking observations of the Yoshiki Sasai group. These initial studies demonstrated the ability of mouse ESCs to directly differentiate into telencephalic precursors in a serum-free suspension culture (SFEB culture). Treatments with WNT and Nodal antagonist resulted in a high yield of PAX6+ cells that could be further differentiated into ventral or dorsal fate after WNT3a or SHH, respectively (Watanabe et al., 2005). Optimization of the SFEB culture media by the inclusion of a BMP inhibitor and the introduction of Y-27632, the selective Rho-associated kinase (ROCK) inhibitor, allowed to translate these findings to human derived systems (Watanabe et al., 2007).

The derivation of optimized mouse and human culture media allowed for the generation of three-dimensional aggregates that recapitulate embryonic corticogenesis and regional specification (Eiraku et al., 2008). Polarized cortical neuroepithelia resembling neural rosettes positive for the expected markers were observed in the floating aggregates. Moreover, cortical specification was obtained, mimicking early corticogenesis with the segregation of discrete layers containing radial glia, neuronal progenitors, and early neurons (Eiraku et al., 2008; Mariani et al., 2012; Kadoshima et al., 2013). Additionally, gene expression profile in these aggregates correlate with the embryonic telencephalon, specifically to the transcriptional program active at 8–10 weeks after conception (Mariani et al., 2012).

Cerebral organoids can be generated via undirected or directed differentiation. The undirected differentiation technique was described by the Knoblich group in 2013 (Lancaster et al., 2013). Single cell hPSCs were aggregated in a serum free media and then embedded in an extracellular matrix (ECM). The presence of ECM supports the formation of neuroepithelial buds that expand into cortical structures under constant agitation (Lancaster et al., 2013; Lancaster and Knoblich, 2014; Renner et al., 2017). The lack of external signaling to induce patterning allows for the formation of organoids with various brain region identities and non-neural derivatives (Camp et al., 2015; Quadrato et al., 2017). Although variable, undirected organoids allow for a high degree of diversity in the cultures that serve as a platform to explore the CNS diversity and the effects of diseases onto different cell types (Lancaster et al., 2017; Paşca, 2018; Kanton et al., 2019; Velasco et al., 2019).

Directed differentiation uses small molecules to induce regional specification (Kadoshima et al., 2013). The absence of ECM, as well as static conditions, allows for the formation of spherical cultures that can be further differentiated into dorsal and ventral aggregates (Paşca et al., 2015; Birey et al., 2017; Sloan et al., 2018), enriched with astrocytes (Sloan et al., 2017) or oligodendrocytes (Marton et al., 2019). Long term culture of these spheroids allows for the continuous maturation and

differentiation of the structures allowing for the exploration of brain development in mid-fetal stages (Paşca et al., 2015; Sloan et al., 2017; Yoon et al., 2019; Gordon et al., 2021). Regional patterning can also be achieved using shaking conditions such as miniaturized bioreactors to generate forebrain, midbrain and hypothalamus organoids (Qian et al., 2016, 2018).

### Challenges and Limitations of Induced Pluripotent Stem Cells Models

Although a remarkable system to study neural development, iPSC-derived models present a set of limitations: cellular variability, lack of maturation, and limited reproduction of the brain cellular complexity (Dolmetsch and Geschwind, 2011; Volpato and Webber, 2020; Yamanaka, 2020; McTague et al., 2021; Qian and Tcw, 2021). These limitations must be considered when translating findings to human development.

The generation of NPCs relied on the differentiation of the hPSCs into neuroectodermal fate (Dhara and Stice, 2008; Chambers et al., 2009), which continues into a preferential acquisition of dorsal forebrain identity (Watanabe et al., 2005; Nat et al., 2007; Zhang M. et al., 2018). Supplementation of exogenous SHH is necessary for a ventral fate acquisition (Ribes and Briscoe, 2009; Liu et al., 2013; Maroof et al., 2013; Nicholas et al., 2013; Tao and Zhang, 2016), as the default dorsal identity is partially due to NPC expression of WNT ligands (Li et al., 2009). Moreover, variability in the capacity of the cells to generate NPCs based on its embryonic or induced origin has been reported (Koyanagi-Aoi et al., 2013), but a more in depth proteomic and genomic characterization has not been done.

Similarities of iPSC-derived cortical neurons to primary cortical neurons have been established through single cell analysis but, layer-specific subtype characterization remains challenging (Handel et al., 2016). NPC cultures are heterogeneous, with mixed populations of neural stem and progenitor cells during the first stages of differentiation. At later stages, combinations of neuron and astrocyte populations can be obtained in a stochastic manner (Hoffman et al., 2017, 2019). This can affect not only the functionality of each subtype but their maturation stage (Brennand et al., 2015). A cleaner, more mature neuronal population can be obtained using direct differentiation methods (Mertens et al., 2018; Meijer et al., 2019; Rhee et al., 2019), yet transfection and selection stress can influence the quality of the cell types that are generated.

Generation of astrocytes and oligodendrocytes is also challenging. Glial cell types appear later in development and require changes in the developmental cues. Current protocols, especially for oligodendrocyte generation, require the use of multiple small molecules and morphogens as well as month-long maintenance to generate cell populations suitable for experimentation (Douvaras et al., 2014; Douvaras and Fossati, 2015; Tcw et al., 2017).

Due to the lack of vascularization, brain organoids display limited growth (Lancaster et al., 2013). Brain organoids generate neurons positive for the six different cortical layers, but layer organization as well as axonal projection patterns lack the expansion seen in similar developmental stages. Moreover, certain structures that are clearly delimited in the developing

brain -such as the subplate and the cortical plate- are difficult to differentiate (Qian et al., 2019). Likewise, intrinsic generation of non-neural cell types such as microglia, vascular cells, and immune cells; requires co-culturing conditions.

Another limitation of the brain organoid system is the inability to recapitulate developmental gradients. Fusion of regional aggregates has been useful to study neural migratory patterns and complex interregional interactions (Bagley et al., 2017; Birey et al., 2017; Xiang et al., 2017, 2019; Miura et al., 2020), but this approach requires the individual generation of the brain regions. The main hurdle to overcome is the correct localization and concentration of morphogens. A pioneering study by the Studer laboratory generated a SHH ventralizing gradient by utilizing a two-step EB formation process (Cederquist et al., 2019). First, a doxycycline-inducible hPSC line capable of expression of the morphogen was used. Sequentially, additional hPSCs were seeded around the SHH signaling EB, creating an organizing center. This approach successfully produces telencephalic organoids with a dorsal-ventral axis. Organoid-on-chip approaches are also being explored. Morphogen-soaked beads positioned near developing cerebral organoids have shown the effects of WNT and BMP4 gradients (Ben-Reuven and Reiner, 2020). WNT inhibition and BMP4 induction generated changes in the transcriptional profile of the areas proximal and distal to the bead, in a concentration dependent manner. These results suggest that recapitulating developmental morphogen gradients may require a combined approach between engineering and developmental biology. The brain organoids system recapitulates some aspects of human CNS development and complementation with other model systems and approaches can expand their capability and potential.

## MITOCHONDRIAL HOMEOSTASIS AND NEURAL DEVELOPMENT

Mitochondria are ubiquitous and essential organelles for cell survival. Known primarily for their capacity to generate energy via oxidative phosphorylation (OXPHOS) and their role in cell death, mitochondria also act as a signaling hub and coordination center for a myriad of cellular processes including metabolite synthesis and calcium buffering. Mitochondrial network remodeling, through fusion and fission, is necessary for discarding damaged or not functional mitochondria (mitophagy), to adapt to new energetic requirements, and to redistribute the organelle throughout the cytoplasm (motility) (Anesti and Scorrano, 2006; Chan, 2012; Cogliati et al., 2013; Schwarz, 2013; Barnhart, 2016; Giacomello et al., 2020). Studies on the involvement of mitochondrial function and dynamics in neurogenesis have been limited by the availability of model systems. The advent of human iPSC-derived systems has opened the possibility to examine the contribution of mitochondrial dynamics, morphology, and function, to cortical development.

### Mitochondria Form and Function Bioenergetics

The energy producing machinery of the mitochondria is located in the cristae, invaginations of the inner mitochondrial

membrane that increase the surface area (Cogliati et al., 2016). The proteins responsible for OXPHOS comprise four different complexes that assemble further into supercomplexes (Schägger and Pfeiffer, 2001; Dudkina et al., 2005; Acín-Pérez et al., 2008). These complexes couple the oxidation of reducing molecules, such as NADH and FADH<sub>2</sub>, to the translocation of protons across the inner membrane. This influx of protons generates a proton electrochemical gradient that is used by the ATP synthase to generate ATP from ADP.

All five complexes are localized along the cristae membrane with an overlapping distribution (Wilkins et al., 2013). The formation of supercomplexes optimizes electron transport and proton shuttling through the inner membrane (Acín-Pérez et al., 2008; Letts and Sazanov, 2017). ATP synthase is localized on the edges of the cristae forming dimers, with the other complexes located along both sides (Davies et al., 2011). This configuration seems to be fundamental for the creation of the proton gradient that accumulates in the concave side of the cristae (Strauss et al., 2008; Rieger et al., 2014).

The correct formation and maturation of the cristae is considered a hallmark of cellular maturation and differentiation. PSCs have fragmented mitochondria with immature cristae (Rafalski et al., 2012) and while oxidative phosphorylation still occurs, they rely preferentially on glycolysis for energy production (Piccoli et al., 2005; Chung et al., 2010; Prigione et al., 2010; Zhang et al., 2011). The differentiation of stem cells into neural stem cells is coupled to metabolic shifts that are essential. Inability to transition from glycolysis to OXPHOS during neural induction causes cell death in ESCs and iPSCs (Zheng et al., 2016; Beckervordersandforth et al., 2017), and inhibition of mitochondrial function blocks neural differentiation and promotes pluripotency (Pereira et al., 2013).

The glycolysis-OXPHOS modulation impacts neurogenesis at different stages. Disruption of OXPHOS-related genes in *Drosophila* inhibits the cell cycle exit and promotes proliferation (Homem et al., 2014; van den Aamele and Brand, 2019). Dysregulation of the mitochondrial complex I function has severe impacts in the capacity of NPCs to proliferate, differentiate into mature neural and oligodendrocytic lineages (Cabello-Rivera et al., 2019). Proliferating intermediate progenitor cells rely on OXPHOS as its main source of energy by downregulation of key glycolytic enzymes and upregulation of enzymes from the TCA cycle and the mitochondrial supercomplexes (Beckervordersandforth et al., 2017).

Although reduced, glycolysis is vital for the NPC population during development. For example, methylglyoxal, a byproduct of glycolysis, influences NPC self-renewal by binding to GAPDH and modulating the translational control of Notch1 (Rodrigues et al., 2020). Other pathways such as glutaminolysis, process by which glutamine is converted into TCA cycle metabolites, have been also associated with cortical expansion and neurogenesis (Rosenberg et al., 2002; Lindhurst et al., 2006; Journiac et al., 2020; Namba et al., 2020).

The mitochondria are also signaling organelles central to the production of TCA cycle metabolites, such as citrate and oxaloacetate, that can then generate macromolecules including lipids and nucleotides (DeBerardinis and Chandel, 2020;

Martínez-Reyes and Chandel, 2020; Chakrabarty and Chandel, 2021) and downstream contribute to histone changes required for neurogenesis (Yang et al., 2019; Shan et al., 2020). Fatty acid metabolism is another aspect related to mitochondrial function that affects neurogenesis. Gain-of-function mutations in the enzyme fatty acid synthase causes reduced proliferation of NPCs in human-derived cerebral organoids (Bowers et al., 2020). Mitochondrial fatty acid  $\beta$ -oxidation (FAO) is also implicated in embryonic neurogenesis. FAO downregulation in mouse cortex decrease self-renewal and apoptosis of NPCs and suggest a key role for FAO in the NPC to IPC transition (Xie et al., 2016). Increase in FAO due to mitochondrial damage can also impair progenitor maturation in mouse neuroblasts (Audano et al., 2019) and striatal progenitors (Warren et al., 2017).

The mitochondria also mediate the release of signaling molecules from the mitochondria (e.g., acetyl-coA, cytochrome c, and free calcium) that control cell fate and function (Martínez-Reyes and Chandel, 2020). Endogenous generation of reactive oxygen species (ROS) in the mitochondria has been shown to promote neurogenesis via NRF2 and Notch pathway inhibition (Khacho et al., 2016), whereas ROS regulation in the developing forebrain via the mitochondrial uncoupling protein 2 (UCP2) is required to induce differentiation of the progenitor pool (Ji et al., 2017). REDOX (reduction-oxidation) balance has been implicated in different aspects of differentiation and neuronal fate acquisition, particularly via Sirt1 and chromatin remodeling (Prozorovski et al., 2008; Tiberi et al., 2012; Bonnefont et al., 2019).

These aspects of mitochondrial signaling beyond ATP production are certainly crucial during the cellular transitions that underlie human brain development. Brain organoids provide a useful tool to gain a mechanistic understanding of the involvement of these unique mitochondrial signaling pathways in human neurogenesis.

## Mitochondrial Dynamics and Remodeling

The active remodeling of the mitochondrial network is crucial for the homeostatic and metabolic adaptation of the cell. Mitochondria are highly dynamic organelles that undergo fission and fusion event according to the cellular and environmental necessities. These highly conserved processes are regulated by large dynamin-related GTPases (Chen and Chan, 2004; Praefcke and McMahon, 2004; Hoppins et al., 2007; Chan, 2012).

Mitochondrial fission or fragmentation is executed by the highly conserved protein Dynamin-related protein 1 (DRP1) (Otsuga et al., 1998; Smirnova et al., 1998; Labrousse et al., 1999). Activation of DRP1, by multiple post-translational modifications, is required for its function at the outer mitochondrial membrane (Taguchi et al., 2007; Han et al., 2008; Chang and Blackstone, 2010; Chou et al., 2012; Prieto et al., 2016). At this site, DRP1 binds to adaptors located at the outer mitochondrial membrane. Mitochondrial fission 1 (FIS1), mitochondrial fission factor (MFF), and mitochondrial dynamics protein 49/51 (MID49/MID51) have been shown to be involved in DRP1 mediated fission (Mozdy et al., 2000; Tieu et al., 2002; James et al., 2003; Griffin et al., 2005; Gandre-Babbe and Van Der Bliek, 2008; Otera et al., 2010; Palmer et al., 2013; Shen et al., 2014;

Liu and Chan, 2015; Osellame et al., 2016). DRP1 self-assembles in rings around the mitochondrial membranes where undergoes conformational changes mediated by GTP hydrolysis and constricts the organelle until it divides (Ingeman et al., 2005; Mears et al., 2011).

Mitochondrial fusion occurs when the outer and inner mitochondrial membrane merge with the corresponding membranes on another mitochondrion. Both fusion events are coordinated and occur simultaneously, resulting in the mixing of the mitochondrial contents in the matrix, membranes and intermembrane space and the homogenization of the mitochondrial DNA (mtDNA) and the formation and assembly of the electron transport chain (ETC) (Chan, 2012). Although coordinated, the fusion of the membranes is directed by distinct mechanisms. Mitofusin 1 (MFN1) and Mitofusin 2 (MFN2) are the proteins responsible for the outer membrane fusion, while optic atrophy 1 (OPA1) mediates inner membrane fusion (Alexander et al., 2000; Delettre et al., 2000; Santel and Fuller, 2001; Rojo et al., 2002; Chen et al., 2003). MFN1 and 2 can homo- or hetero-dimerize with mitofusins in the adjacent mitochondria. OPA1 have two proteolytically cleaved proteins: long OPA1 (OPA1-L) and short OPA1 (OPA1-S). OPA1-L is anchored in the inner mitochondria membrane and coordinated the fusion process by forming homodimers with the opposite target membrane. OPA1-S has been associated with the stabilization of the mitochondrial cristae, maintenance of the mtDNA content and energetic competence (Mishra et al., 2014; Del Dotto et al., 2017).

Neurodevelopmental studies have shown mitochondrial dynamics are essential in neurogenesis. Mouse models deficient in DRP1 show smaller brain size and reduced developmental apoptosis in the neural tube (Wakabayashi et al., 2009; Lewis et al., 2018); as well as high mortality of newborn deep layer neurons (Ishihara et al., 2009). Although rare, mutations in DRP1 have been identified in patients with severe neurodevelopmental delays (Baum and Gama, 2021). Mutations in the adaptor proteins MFF and MID49 have also been associated with developmental delay, myopathies, and neuropathies (Shamseldin et al., 2012; Koch et al., 2016; Bartsakoulia et al., 2018; Baum and Gama, 2021). Mutations in MFNs and OPA1 have also identified as the causal mechanism behind neurodegenerative diseases such as Charcot-Marie-Tooth syndrome, autosomal dominant optic atrophy, spastic paraplegia, syndromic Parkinson and dementia (Züchner et al., 2004; Alavi et al., 2007; Verny et al., 2008; Yu-Wai-Man et al., 2010; Carelli et al., 2015).

Studies in mouse models have shown significant cristae structural changes at the end of neural tube closure, as cells progress into neural progenitor stage. This modification in the mitochondria morphology correlates with the transition from a highly glycolytic metabolism to an OXPHOS dependent one (Fame et al., 2019). This switch in the mitochondrial structure, and the subsequent energetic requirements, are dependent on the downregulation of MYC, which has been shown to be a key regulator of ribosomal biogenesis, protein synthesis, and cellular proliferation at this stage (Chau et al., 2018). In vitro differentiation of mouse cortical neurons causes changes in mitochondrial mass due to increase mitochondrial biogenesis

mediated by upregulation of mitochondrial transcription factor A (TFAM) and peroxisomal proliferating activating receptor  $\gamma$  coactivator-1 $\alpha$  (PGC-1 $\alpha$ ) (Agostini et al., 2016). Glycolysis-to-OXPHOS transition was also observed during neuronal differentiation.

Disruption of mitochondrial fission and fusion proteins has been shown to result in both neurodevelopmental and neurodegenerative disease, both age-associated and progressive (Khacho and Slack, 2018). Mitochondrial dysfunction and aberrant mitochondria morphology are hallmarks of impaired brain development in both animal and human derived models (Waterham et al., 2007; Ishihara et al., 2009; Wakabayashi et al., 2009; Fang et al., 2016; Spiegel et al., 2016).

The regulation of mitochondrial dynamics and its physiological relevance in the context of tissue architecture is still an unexplored landscape (Liesa and Shirihai, 2013; Noguchi and Kasahara, 2017). Studies of the mitochondrial morphology in adult mouse brain have shown that different cellular stages of differentiation possess a distinct mitochondrial morphology. Adult hippocampal radial glia-like NSCs have mixed globular and tubular shape mitochondria, while IPCs have more thin and more elongated network and adult neurons featured a wider and highly elongated morphology (Beckervordersandforth et al., 2017). Neurons also have higher mitochondrial volume, which can be correlated with an increase in bioenergetics mediated by the ETC and OXPHOS activity (Bélangier et al., 2011).

In the developing mouse brain, Khacho et al. (2016) described the morphology of the mitochondria at different cortical compartments. Neural stem cells in the VZ, positive for the marker SOX2, presented an elongated mitochondrial network; while IPC, stained with the marker TBR2, in the SVZ have a fragmented morphology. Newly committed neurons expressing TUJ1 ( $\beta$ III-tubulin), regained an elongated mitochondria structure in the cortical plate. Deletion of OPA1 and MFN1/2, GTPases that mediate mitochondrial membrane fusion, impaired neural stem cell self-renewal and promote early differentiation as a result of the sustained mitochondrial fragmentation. Promotion of highly fused mitochondria by manipulation of DRP-1, the main effector of mitochondrial fission, had the opposite effect by increasing the ability of neural stem cells to self-renew.

In a follow up study, Iwata et al. (2020) analyzed the changes of the mitochondrial network through the early stages of neurogenesis in 2D cultures of mouse and human neural cells. From their results, PAX6+ RGC displayed fused mitochondria and TBR2+ IPCs had an intermediate mitochondrial size. In their case,  $\beta$ III-tubulin expressing neurons had a fragmented mitochondrion. Interestingly, they showed that the cell fate specification occurs during a restricted time window during the postmitotic period where daughter cells inheriting fragmented mitochondria differentiate and daughter cells inheriting a fused network will retain the capacity to self-renew.

Although we are beginning to elucidate the role of mitochondria morphology and homeostasis during neuronal specification, little is known in human models about the role that these organelles play during gliogenesis. Astrocytes rely on glycolysis more than OXPHOS for their energy production,

specially by the utilization of fatty-acids as source of fuel (Eraso-Pichot et al., 2018; Fecher et al., 2019; White et al., 2020). Moreover, developing astrocytes contain a highly interconnected functional network of mitochondria and upregulation of the mitochondrial network is crucial for coordinating post-natal astrocyte maturation and synaptogenesis (Zehnder et al., 2021).

Oligodendrocytes, on the other hand, rely on OXPHOS during the progenitor stage and switch to glycolysis when mature (Rinholm et al., 2011; Fünfschilling et al., 2012; Amaral et al., 2016; Rao et al., 2017; Fecher et al., 2019). Mitochondrial fragmentation is key in mature oligodendrocytes as myelin sheets contained smaller mitochondria if compared to the network surrounding the nucleus (Rinholm et al., 2016; Battefeld et al., 2019). Yet, the effects of the mitochondrial dynamics during their development and specification remains unknown.

## Mitochondria Morphology Changes Through Neural Differentiation and Maturation

Due to the highly dynamic nature of mitochondria, analysis of its morphology has been challenging in *in vivo* and 3-D settings. Most of the existing classification have resorted to manual and morphological classification (e.g., fused vs. fragmented) of the mitochondrial networks, utilizing qualitative or semi-quantitative approaches (Rafelski, 2013; Prieto et al., 2016; Noguchi and Kasahara, 2017; Faltg et al., 2021; Fogo et al., 2021). Advances in imaging techniques coupled with computational approaches have improved the capacity to unbiasedly and consistently assess the morphology of these organelles (Leonard et al., 2015; Zahedi et al., 2018). Recently, machine learning algorithms (Kan, 2017) together with genetic perturbations of key mitochondrial players, have been proposed as a potential alternative to not only evaluate the phenotypical aspects of the mitochondria but to assess the physiological relevance of those changes (Fogo et al., 2021).

A comprehensive study of the mitochondrial network of different cell types during early neurogenesis is required. In accordance with previous findings in the mouse brain and *in vitro* neurons (Khacho et al., 2016; Iwata et al., 2020), NPCs that are positive for the transcription factors PAX6 and SOX2 show an elongated mitochondrial network. In contrast, committed neurons positive for the cytoskeletal marker  $\beta$ III-tubulin have a fragmented network. While this characterization is semi-quantitative, recent tools using machine learning have been developed that can be coupled to refine the analysis of the mitochondrial morphologies in different cell types (Berg et al., 2019). This analysis could make it possible to compare the changes observed in mouse brains and 2-D hPSC-derived neurons to a 3-D model of development and shed light into the differences and similarities of the different research models. This new method of mitochondrial scoring and quantification can be extended to other neural organoid protocols. For example, dorsal and ventral spheroids (Birey et al., 2017) or thalamic organoids (Xiang et al., 2019) can be used to explore the effects of mitochondrial and mitochondrial associated mutations in the migration of axons. This would be of particular interest in

disease models where can be axonal migration can be affected or where proper formation of axonal tract play a crucial role in the pathophysiology of the disease (Giandomenico et al., 2019; Kitahara et al., 2020). These techniques can be also used for the characterization of the mitochondrial morphology and the network regulation in other tissues during development and disease. Organoids that mimic highly metabolic tissues such as cardiac muscle, kidney and liver could be used to understand the effects of the mitochondrial dynamics under homeostatic and allostatic conditions.

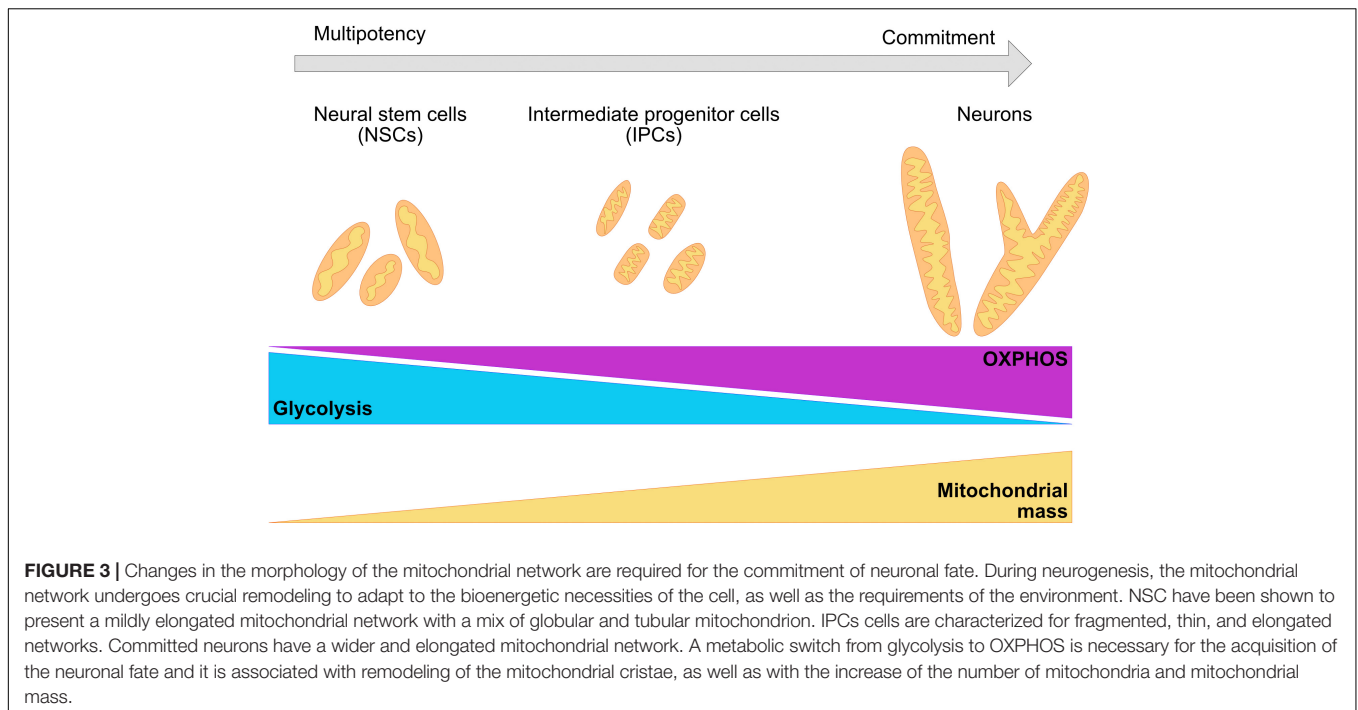
## MITOCHONDRIAL FITNESS IN NEUROGENESIS AND DISEASE

Mitochondrial function is central to the homeostasis of highly metabolic tissues. The brain is responsible of consuming nearly 20% of the oxygen and calories from the body, while representing about 2% of its total weight (Raichle and Gusnard, 2002; Picard and McEwen, 2014). Although mitochondrial dysfunction cause by mutations in mitochondrial or metabolic genes results in severe multisystemic disease, the brain is more vulnerable to these defects in mitochondrial function. Thus, mitochondrial health sustains the functional and structural plasticity of the CNS.

The exact mechanisms underlying the regulation of mitochondrial dynamics during human neural development have remained widely unexplored, as most studies have been done in yeast, cultured mammalian cells, and mice (Liesa and Shirihai, 2013; Noguchi and Kasahara, 2017). As mentioned previously the known differences between human and mouse brains (Arbour et al., 2008; Pressler and Auvin, 2013; Khacho et al., 2016; Khacho and Slack, 2018) make studies in human models imperative. Whether mitochondrial function in bioenergetics, calcium handling, ROSs production, and other signaling events, differ among human neural populations and what is the contribution of mitochondrial fitness during the neuronal specification, migration, synaptic transmission, and cognition, could be revealed using human models (Ioannou et al., 2019).

Remodeling of the mitochondrial network as cells commit to a neuronal cell fate is crucial for survival and function (Figure 3; Chan, 2012; Schwarz, 2013; Khacho et al., 2016; Khacho and Slack, 2018; Iwata et al., 2020). Landmark studies demonstrate that modulation of mitochondrial dynamics during a post mitotic period can change the number of NPCs or neurons that are being produced in both mouse brains and hESC derived neurons (Hara et al., 2014; Iwata et al., 2020). In murine models, the mitochondrial network transitions from elongated structures in neural stem cells to fragmented mitochondria in intermediate progenitor cells and back to elongated structures in mature neurons (Khacho and Slack, 2018; Iwata et al., 2020). It is currently not clear whether these dynamic changes in mitochondrial shape are maintained in the human brain and their involvement in maintaining the metabolic profile of the neurons at different stages of differentiation and maturation.

Revealing the molecular underpinnings of mitochondrial form and function during the early stages of neurogenesis is fundamental to developing therapies that may control



human disease. Coupling human brain organoids to super resolution microscopy, optogenetic approaches, gene editing and other technical approaches could uncover the role of mitochondria in the regulation of neurogenesis, synaptic transmission, brain function, and cognition. In the next sections, we provide an overview of recent advances in understanding the intricate relationship between mitochondrial function and brain development that have result from using human iPSC brain models.

The ability of studying mitochondrial fitness during early development could allow for the understanding of other neurodevelopmental disease caused by environmental factors and maternal health. The organoid system could be used to determine the effects of metabolic stress and nutrient imbalance in the developing brain as maternal metabolic diseases has been shown to correlate with increased risk of neurodevelopmental and psychiatric diseases in both human and animal studies (Shook et al., 2020; Edlow, 2021). Also, the capacity to recapitulate the formation of the neural tube as neural rosettes or as neuruloids -self-organizing structures containing neural progenitors, neural crest, sensory placode and epidermis- (Haremaki et al., 2019) could allow for the exploration of the molecular and cellular mechanisms behind complex CNS birth abnormalities.

### Mutations Associated With Neurodevelopmental Diseases Disrupt Mitochondrial Morphology and Function in Cerebral Organoids

Inborn errors of metabolism are rare genetic disorders resulting from defects in metabolic pathways (Das et al., 2010;

Agana et al., 2018). Mitochondrial diseases are the most common group of inherited metabolic disorders and are among the most common forms of inherited neurological disorders (Gorman et al., 2016). These illnesses involve multiple organ systems and have limited therapeutic options (Parikh et al., 2017; Grier et al., 2018; Schaefer et al., 2019).

Leigh syndrome (LS) is one of these rare inherited neurometabolic diseases. Mutations in more than 75 genes associated with ATP production have been identified as causal, both in nuclear and mitochondrial DNA. It affects mostly infants within their first year of life and has a poor prognosis and a low survival expectancy (Finsterer, 2008; Lake et al., 2016). It is characterized by abnormal motor findings, epileptic seizures, increased lactate in the blood and cerebrospinal fluid, failure to thrive, and focal, bilaterally symmetrical necrotic lesions in the brain (Sofou et al., 2014, 2018). As it is a highly heterogeneous disease, the establishment of animal and in vitro models has been challenging and limited to only select mutations.

Animal models have been used to test therapeutic approaches, with mixed results. Gene editing using adeno-associated virus in *Ndufs4*<sup>-/-</sup> mice has shown partial rescue of the phenotype (Di Meo et al., 2017). Supplementation of nicotinamide riboside to *Sco2*<sup>-/-</sup> mice showed improvement of the respiratory chain defect and increased exercise tolerance due to improved mitochondrial biogenesis (Cerutti et al., 2014). Hypoxia and low oxygen availability in the brain have also been shown to increase the life span and improve neurological findings in *Ndufs4*<sup>-/-</sup> mice (Jain et al., 2016, 2019; Ferrari et al., 2017).

Reprogramming of patient fibroblast harboring nuclear and mitochondrial mutations (Galera-Monge et al., 2016; Zurita-Díaz et al., 2016; Grace et al., 2019; Romero-Morales et al., 2020; Inak et al., 2021; Meshrkey et al., 2021) has been used

to generate specialized cells for the study of the impact of LS-associated mutations in highly metabolic tissues. Human iPSC models have been proposed as platforms to test new therapeutic approaches such as somatic nuclear transfer (Ma et al., 2015). Direct reprogramming of fibroblasts into neurons has been used to overcome the effects of heteroplasmy during reprogramming and as an alternative for targeted high-throughput drug screening and advancing precision medicine (Villanueva-Paz et al., 2019; Villalón-García et al., 2020).

These approaches have proven useful to investigate the effects of LS causing mutations in early neural development (Romero-Morales et al., 2020; Inak et al., 2021). Inak et al. (2021) generated iPSCs with mutations in the complex IV assembly gene *Surfeit locus protein 1 (SURF1)* and CRISPR/Cas9 corrected lines. As early as NPCs, deficiencies in the ability to switch to an OXPHOS energetic program and failure to differentiate into neurons were observed in both monolayer and brain organoid cultures. Aberrant cytoarchitecture was also observed in the LS mutant organoids. Importantly, rescue with *SURF1* gene augmentation was able to rescue the observed phenotype, as well as pharmacological induction of PGC1A via bezafibrate, an antilipemic agent that lowers cholesterol and triglycerides (Behar et al., 2000). The second study used three commercially available cell lines derived from patients with LS (Romero-Morales et al., 2020). By whole exome sequencing and mitochondrial sequencing, the putative mutations were identified in the nuclear genes pyruvate dehydrogenase (PDH) in two of the cell lines, and dihydrolipoyl dehydrogenase (DLD); as well as the mitochondrially encoded ATP synthase membrane subunit 6 (MT-ATP6). Although not major differences were observed at the NPCs level, three-dimensional cultures showed significant deficiencies in neuronal commitment and self-organization. The double mutant cell line MT-AP6/PDH showed aberrant organization of the neuroepithelial bud formation as early as day 10 in the brain organoid protocol. These deficiencies were more apparent at day 30 where the loss of the stereotypical luminal and VZs was lost. Moreover, qPCR analysis of the MT-AP6/PDH mutant organoids show an increase in expression of the intermediate progenitor cell marker TBR2. Analysis of the mitochondria in the SOX2+ cells in day 30 brain organoids showed aggregated mitochondrial network in the double mutant in comparison with the elongated mitochondria observed in the control. Metabolic deficiencies were also identified at this stage suggesting that the mutant cells rely on aerobic glycolysis or Warburg effect for survival. The inability to switch to a OXPHOS energetic program may also contribute to the effects seen later in day 100 organoids where the neuronal population in all LS-patient derived organoids was significantly reduced.

The profound dysregulation of corticogenesis in the LS-patient derived organoids may suggest that there is an underestimation of the prevalence of the disease in the population (Feeney et al., 2019). Prenatal genetic evaluation is now being performed for cases where there is a known risk for mitochondrial mutations (White et al., 1999; Craven et al., 2017; Schubert and Vilarinho, 2020). This testing can be performed as early as 10–12 weeks post conception and is usually

requested if there is a history of a previously affected child or first-degree relative (Nesbitt et al., 2014). Interpretation of these tests is challenging in the case of mitochondrial mutations due to heteroplasmy; the biopsied tissues may exhibit a different mutational burden compared with other fetal tissues (Harding et al., 1992; Ferlin et al., 1997; Steffann et al., 2007; Nesbitt et al., 2014).

The use of iPSCs and iPSC-derived models can become a platform for research and therapeutic testing as diagnosing and treating this disease remains a challenge due to its heterogeneity and complexity. Work in mice has shown that chronic hypoxia can prevent and even reverse some of the neurological findings in LS (Jain et al., 2019), but these have not been tested in human models yet. The use of brain organoids may be an appropriate tool to test whether the reduction of oxygen tension can rescue the LS phenotype and to test for other therapeutic approaches that could alleviate the disease onset and progression. Specifically for the organoids system, there is the capacity to produce individual regions of the brain and investigate the effects of the LS causing mutations in the neuronal subpopulation. Moreover, assembloids (Paşca, 2018; Sloan et al., 2018; Miura et al., 2020) can be generated to investigate the interactions between these regions; specifically the metabolically demanding interneuronal migration that occurs from the ventral forebrain to the dorsal forebrain. Errors in myelination and formation of the axonal bundles can also be an avenue of interest as oligodendrocyte loss has also been observed in LS (Anzil et al., 1981; Pliss et al., 2004; Marton et al., 2019). Also, long term maintenance (> 100 days) of brain organoids can be used to understand the role of astrocytes in the pathophysiology of LS.

## Mitochondrial Apoptosis and Neural Cell Specification

Mitochondria are at the crossroads of cell death and metabolism (Rastogi et al., 2019). The BCL-2 family of proteins regulates cell death at the mitochondria (Knudson et al., 1995; Hsu et al., 1997; Kluck et al., 1997, 1999; Vander Heiden et al., 1997; Inohara et al., 1998; Jürgensmeier et al., 1998; Green, 2000; Wei et al., 2000; Ke et al., 2018) and has been implicated in maintaining mitochondrial homeostasis in the absence of a cell death signal (Li et al., 2008, 2013; Rasmussen et al., 2018, 2020; Salisbury-Ruf et al., 2018; Joshi et al., 2020). Programmed cell death (apoptosis) is an integral part of brain development and maturation (Kuan et al., 2000). Immature human brains have nearly 50% more neurons than adults, as many neurons die by apoptosis during normal brain morphogenesis and neuronal histogenesis. Apoptosis is assumed to prevent overgrowth of the neuroepithelium by controlling neural cell numbers in the developing brain (Kuan et al., 2000; Akhtar et al., 2004; Nonomura et al., 2013; Yamaguchi and Miura, 2015). Reduced cell death in mice bearing mutations in pro-apoptotic molecules (e.g., caspase 3, caspase 9) result in severe brain malformations including indented neuroepithelium, compressed brain ventricles, and neural tube closure defects (Nonomura et al., 2013). Additionally, in early brain development, massive cell death is restricted to specific

areas, suggesting that local apoptosis might affect the gross organization of the developing organ (Kuan et al., 2000; Akhtar et al., 2004).

Previous studies showed that BAX/BAK deficient cells have some defects in mitochondrial morphology (Karbowski et al., 2006). BAX also is known to colocalize with the primary executioner of mitochondrial fission Dynamin related protein-1 (DRP-1) during early stages of apoptosis (Karbowski et al., 2002). Thus, BAX/BAK are also potential regulators of mitochondrial homeostasis during development. BAX/BAK CRISPR knock-out iPSCs were recently used to study the function of the BCL-2 family in early neurogenesis (Joshi et al., 2020). Day 30 brain organoids generated from the control lines showed the expected markers and architecture for this developmental timepoint. hNPCs stained with PAX6, SOX2 and NESTIN formed the VZ/SVZ around the lumen-like structure. oRGs that were TBR2 positive decorated the edge of the VZ/SVZ, and cortical plate cells positive for TBR1 and CTIP2 were observed Joshi et al. (2020). In contrast, BAX/BAK DKO brain organoids showed a profound loss of cortical organizations and a reduction in the cells positive for the aforementioned markers (Joshi et al., 2020). Analysis of the areas of the organoids that were positive for the NPC marker SOX2, showed aggregation of the mitochondrial network in the DKOs. Control organoids showed a conserved organization of the VZ/SVZ and the expected elongated network for SOX2+ NPCs. This loss of neural markers in conjunction with the disruption of the mitochondrial network in the DKO may suggest that BAX and BAK are fundamental factors maintaining mitochondrial morphology, and their absence generate a downstream effect in the specification of the cell fate. Additional studies are needed to determine the exact mechanisms by which homeostatic regulation of apoptosis controls neurogenesis.

BAX/BAK KO iPSCs allow for the possibility to use this genetic background for the study of deleterious mutations than otherwise would cause cell death. It could also serve as a platform for the study of developmental apoptosis, and the signaling mechanisms that dying cells are involved. Apoptosis is a key regulator for the epithelial-mesenchymal transition (EMT) necessary for the acquisition of cardiac lineage (Fort et al., 2021). EMT is also crucial for the migration of the neural crest cells from the neural tube to the surrounding tissue for the formation of peripheral neurons, melanocytes, craniofacial bones and muscle, among other structures (Hay, 2005).

Particularly in the brain, developmental cell death is crucial for the during development and maturation of the neuronal connections. In the specific case of neuronal pruning, studies suggest that dysregulation of the mitochondrial membrane potential and the production of ROSs are signals for axonal retraction and degradation (Baranov et al., 2019; Lieberman et al., 2019). Yet, it is not known how the mitochondrial dynamics affect these events. Moreover, as apoptotic cells are key for the colonization of the brain by microglia (Arnò et al., 2014; Casano et al., 2016), co-culturing experiments between BAX/BAK KO organoids with microglia can help elucidate the mechanism of microglia migration and proliferation in the developing brain.

## Mitochondria Dysregulation and Hypoxic Injury

Extreme prematurity and complications prior to or during birth can contribute to hypoxic episodes and subsequent brain injury in neonates (Lima et al., 2018). As survival increases, so does the life-long neurological disabilities associated with CNS injury. Mitochondrial energy production is impaired when the tissue oxygen tension is reduced (Santore et al., 2002; Lages et al., 2015). After reoxygenation, mitochondrial respiration transiently resumes but it is later suppressed (Xie and Wolin, 1996; Weidemann and Johnson, 2008; Goda and Kanai, 2012). Mitochondrial hyperpolarization results in the increased production of ROS leading to accumulation of mutations (Grivennikova et al., 2010), decreased metabolic output, and increased susceptibility to cell death (Kudin et al., 2004).

Studies using rat brains showed that the mitochondrial morphology in neurons shifts from elongated to more granular/fragmented appearance after the hypoxic insult (Puka-Sundvall et al., 2000; Hallin et al., 2006). Mitochondrial migration toward the nucleus, as well as accumulation in the perinuclear region, has also been described and associated with the onset of cell death (Northington et al., 2001; Hallin et al., 2006).

Pathological mitochondrial fragmentation has been reported in *in vivo* and *in vitro* models. Loss of the mitochondrial membrane potential leads to the cleavage OPA1 – responsible for inner mitochondrial membrane fusion. Oxygen deprivation increases the amount of a short non-functional form of OPA1, resulting in rapid induction of fission and ultimately cell death (Ehse et al., 2009; Head et al., 2009; Baburamani et al., 2015; Sanderson et al., 2015; Kumar et al., 2016). Interestingly, downregulation of DRP1 can prevent the permeabilization of the mitochondrial membrane and the progression to cell death (Grohm et al., 2012) by potentially preventing cristae remodeling (Ramonet et al., 2013). However, it is not known if these morphological changes, their regulation, and their effects on mitochondrial function are involved during neonatal hypoxia. Prolonged depression in mitochondrial metabolism (Brekke et al., 2017), as well as rapid induction of mitochondrial fragmentation (Demarest et al., 2016), validate the emerging evidence of the mitochondria as central regulators of the hypoxic injury cascade.

Results from animal models have shown that gray and white matter are the most vulnerable areas of the brain to neonatal hypoxia ischemic injury. The cerebral cortex is predominantly affected (Volpe, 2012) and the localization and extent of the insult have been shown to correlate with specific neurodevelopmental symptoms later in life (Gonzalez and Miller, 2006; Steinman et al., 2009). Considering that the spatiotemporal regulation of neuronal proliferation, migration and differentiation varies among mammals, a human derived model is needed to understand species specific variation.

The cerebral cortex is highly sensitive to hypoxic insult. Cell death in this region presents a laminar distribution, especially involving layers III and V (Volpe, 2012). Work in early stages of brain organoid formation have shown that hypoxic stress



causes growth arrest, massive cell death (Daviaud et al., 2019) and reduced expression of the cortical markers TBR1, CTIP2, SATB2, and the astrocyte marker GFAP (Boisvert et al., 2019). In a model of encephalopathy of prematurity, human cortical spheroids resembling the cerebral cortex at midgestation were exposed to 48h of low oxygen tension (<1%) (Paşca et al., 2019). Hypoxia induced a reduction in TBR2+ intermediate progenitor cells and an increase in CTIP2+ cells, suggesting premature neural differentiation (Paşca et al., 2019).

As the mitochondrial network undergoes remodeling in the different cellular niches of the developing brain (Khacho et al., 2016, 2019; Khacho and Slack, 2018), it would be interesting to analyze if the that hypoxic insult affects each neural population differently due to dysregulation of mitochondrial dynamics. Hypoxic insult may affect the mitochondrial network plasticity due to dysregulation of the mitochondrial dynamic's machinery. Rates of fission may increase as OPA-1 undergoes cleavage into short non-functional form (Ehse et al., 2009). The high sensitivity to hypoxia of intermediate progenitor cells may be due to changes in mitochondrial dynamics and morphology. As the intermediate progenitor cells have been proposed to undergo cell death during hypoxic injury, characterization in this context has been challenging. Brain organoid models could also be used to examine other determinants of mitochondrial homeostasis including mitochondrial biogenesis and mitophagy as well as to analyze the link between oxygen deprivation and mitochondrial dysfunction. Mitochondrial disorders can intensify the effects of hypoxia by exacerbating potential deleterious reactions (Volpe, 2012) or diminishing prosurvival mechanisms (Ten and Starkov, 2012). Cell lines generated from mitochondrial diseases could be used to determine how mitochondrial impairment affects the response to hypoxia, as failure of the metabolic machinery has been shown to contribute to brain injury in mouse models (Niatsetskaia et al., 2012). Paradoxically, hypoxia has been shown to reverse some of the effects of mitochondrial disorders such as Leigh syndrome (Jain et al., 2019).

## CONCLUSION

The underlying mechanisms responsible for the extraordinarily complex cognitive capacity of the human brain are beginning to be elucidated. A central principle governing brain development

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is the precise spatiotemporally coordinated birth of, and interactions between, a vast number and types of neurons. Recent studies point to a new mechanism – one hinted at previously, but currently poorly understood. A spectrum of mitochondrial fitness properties could provide internal support to the intrinsic developmental programs of various neuronal types, while also being responsive to environmental and intercellular signals. In humans, defects in mitochondrial homeostasis are linked to conditions such as Leigh syndrome (a neurometabolic disorder), MELAS syndrome (mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes) (a neurodegenerative disorder), and Autism Spectrum Disorder (a neurodevelopmental disease). But, the mechanisms by which mitochondrial morphology and function influence human brain development are largely unexplored.

A massive challenge in the past has been the lack of appropriate model systems that can reproducibly recapitulate the heterogeneous nature as well as the highly complex properties of the human brain. As reviewed here, brain organoids reproduce several key features of human cortical development, including progenitor zone organization, neurogenesis, migration, and synaptic activity. Studies using human brain organoids will continue to provide insight into the mitochondrial mechanisms underlying neurogenesis as well as provide new angles to understand mitochondrial disorders.

## AUTHOR CONTRIBUTIONS

AR-M performed the initial literature review. AR-M and VG wrote the manuscript. Both authors contributed to the article and approved the submitted version.

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