Modeling Complex Neurological Disorders With Human Induced Pluripotent Stem Cells

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Modeling Complex Neurological Disorders With Human Induced Pluripotent Stem Cells

Abstract
The etiology of many complex neurological disorders, including schizophrenia (SZ) and HIV-Associated Neurocognitive Disorders (HAND), are still unknown, partly due to a lack of accurate models. Human-induced pluripotent stem cells (HiPSCs), which can be differentiated into any cell type and capture the genetic variation across humans, may serve as a powerful model to elucidate the mechanisms behind such disorders. In this thesis we have explored mechanistic and cellular phenotypes of the complex disorders Schizophrenia and HAND. First, we studied 22qDS, a hemizygous microdeletion that occurs at chromosome 22q11.2 and leads to complex neuropsychiatric phenotypes including SZ in 25% of 22qDS individuals. Since 6 of the 40 genes deleted in 22qDS encode for proteins that directly localize to mitochondria, we tested the hypothesis that SZ and 22qDS are associated with mitochondria defects. We found that HiPSC-derived neurons from 22qDS+SZ indeed have mitochondria deficits. We further discovered that haploinsufficiency of only the 22qDS gene MRPL40, a component of the mitochondrial ribosome, sufficiently replicated the mitochondria deficits. Another important gene in the 22q region, CLDN5 functions in tight junctions in the blood-brain barrier (BBB). We hypothesized that there would be deficits in HiPSC-derived BBB in 22qDS+SZ. We found impaired BBB function and increased inflammation. Impaired mitochondria function and BBB integrity may synergistically collaborate leading to increased chance of neuropsychiatric development. Third, to study the interactions between HiPSC-derived cell types in a disease process, we focused on the study of HIV, which leads to HAND in over half of HIV-infected individuals despite antiretroviral treatment (ART). We systematically combined independently differentiated neurons, astrocytes, and microglia to generate a tri-culture with or without HIV-infection and ART. We found that infection led to activation of EIF2 in all three cell types, which was largely resolved by treatment with the antiretroviral compound efavirenz (EFZ). However, EFZ treatment enhanced distinct inflammation signatures and TNFa production in HIV-infected microglia. These findings validate the tri-culture and reveal potential therapeutic targets for HAND. In sum, this thesis provides evidence that HiPSCs can be used in complex cultures to study the cellular and molecular mechanisms behind complex neuropsychiatric and neurocognitive disorders.

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MODELING COMPLEX NEUROLOGICAL DISORDERS WITH HUMAN INDUCED PLURIPOTENT STEM CELLS

Sean Kerry Ryan

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Sean Kerry Ryan
To my parents, Regis and Rachel, thank you for your endless support throughout my scientific career.
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ABSTRACT

MODELING COMPLEX NEUROLOGICAL DISORDERS WITH HUMAN INDUCED PLURIPOTENT STEM CELLS

Sean Kerry Ryan
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Kelly L. Jordan-Sciutto, Ph.D.

The etiology of many complex neurological disorders, including schizophrenia (SZ) and HIV-Associated Neurocognitive Disorders (HAND), are still unknown, partly due to a lack of accurate models. Human-induced pluripotent stem cells (HiPSCs), which can be differentiated into any cell type and capture the genetic variation across humans, may serve as a powerful model to elucidate the mechanisms behind such disorders. In this thesis we have explored mechanistic and cellular phenotypes of the complex disorders Schizophrenia and HAND. First, we studied 22qDS, a hemizygous microdeletion that occurs at chromosome 22q11.2 and leads to complex neuropsychiatric phenotypes including SZ in 25% of 22qDS individuals. Since 6 of the 40 genes deleted in 22qDS encode for proteins that directly localize to mitochondria, we tested the hypothesis that SZ and 22qDS are associated with mitochondria defects. We found that HiPSC-derived neurons from 22qDS+SZ indeed have mitochondria deficits. We further discovered that haploinsufficiency of only the 22qDS gene MRPL40, a component of the mitochondrial ribosome, sufficiently replicated the mitochondria deficits. Another important gene in the 22q region, CLDN5 functions in tight junctions in the blood-brain barrier (BBB). We hypothesized that there
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1. Introduction

1.1 Stem Cells in development and by induction

During normal development, the totipotent, fertilized egg loses potency as it develops into specialized cell types that are unipotent. Recent technological advances have allowed researchers to reprogram unipotent cells back into pluripotent stem cells, allowing for directed differentiation into any cell type in the body. Adapted from (Waddington, 1940) and (Hochedlinger & Plath, 2009).

1.1.1 Stem cells in normal development

Stem cells are the basis for all differentiated cell types. From the totipotent, fertilized egg that is capable of producing a full organism, to embryonic, pluripotent stem cells found in the blastocyst that are capable of forming all three germ layers, to multipotent stem cells able to produce several,
related cell types (Figure 1), stem cells have extraordinary potential. Harnessing this biological tool has allowed us to determine the responses of pathologically relevant cell types to drugs for therapy (Kaufmann et al., 2015) and understand diseases through modeling (Lee et al., 2009), with the potential to treat therapeutically difficult or impossible diseases (Kimbrel & Lanza, 2015).

1.1.2 Cultured embryonic stem cells

While mouse embryonic stem cells (ESCs) had been successfully cultured since the early 1980’s (Evans & Kaufman, 1981; Martin, 1981), the first nonhuman primate ESC line was established in 1995 (Thomson et al., 1995). Jeffery Jones’ group first derived human ESCs from blastocysts in 1998 (Thomson et al., 1998). To distinguish ESCs from cancerous embryonal carcinoma cells, three requirements were established: derived from pre- or peri-implanted embryos, extended proliferation with no spontaneous differentiation, and potential to differentiate into all three germ layers (Thomson et al., 1998). As these cells come from aborted fetuses (Thomson et al., 1998), there has been significant controversy over their use (Holm, 2002). In order to avoid such controversies, significant research went into developing pluripotent stem cells from somatic cells.
1.1.3 Induced pluripotent stem cells

Figure 2: Schema for iPSC reprogramming

Somatic cells, usually fibroblasts or lymphocytes, are collected from the donor and transfected with the four transcription factors, OCT4, SOX2, KLF4, and MYC by episomal vectors, sendai virus, or other method. After several weeks, iPSC colonies develop and are isolated.

Nuclear transplantation into oocytes from embryonic and somatic cells leads to pluripotency, showing that factors outside the nucleus can induce gene expression that reprograms the cell into an undifferentiated state (Briggs & King, 1952; Gurdon, 1962; Gurdon, Elsdale, & Fischberg, 1958; Wilm, Schnieke, McWhir, Kind, & Campbell, 1997). Shinya Yamanaka’s group utilized this information and became the first to reprogram fetal and adult somatic cells, specifically fibroblasts, into Induced pluripotent stem cells (iPSCs) in 2006 (K. Takahashi & Yamanaka, 2006). This was quickly followed one year later with iPSCs derived from human cells (HiPSCs) (K. Takahashi et al., 2007; Yu et al., 2007). For both species, Yamanaka’s group utilized a set of four transcriptions factors, OCT4, SOX2, KLF4, and MYC, coined the Yamanaka factors, to reprogram somatic cells into pluripotent cells (Figure 2) (K. Takahashi et al., 2007; K. Takahashi & Yamanaka, 2006). OCT4 and SOX2 help maintain
pluripotency in ES cells and the embryo (Avilion et al., 2003; Niwa, Miyazaki, & Smith, 2000), and KLF4 and MYC are upregulated in tumors (Cartwright et al., 2005; Y. Li et al., 2005), which help sustain proliferation. The second group used a similar set of four transcription factors, OCT4, SOX2, NANOG, and LIN28, to convert human cells into HiPSCs (Yu et al., 2007). Several groups rapidly increased the efficiency of reprogramming and survivability of the iPSCs as well as improved the gene expression to better mimic ESCs by selecting for Nanog expressing iPSC clones and utilizing ROCK inhibitor for massively increased survivability of single cell dissociations (Maherali et al., 2007; Okita, Ichisaka, & Yamanaka, 2007; Watanabe et al., 2007; Wernig et al., 2007).

Cells were initially reprogrammed with integrating viruses such as retroviruses and lentiviruses (K. Takahashi et al., 2007; K. Takahashi & Yamanaka, 2006). However, this created concern for clinical applications (Hawley, 2008). From this concern, several non-integrating strategies have been developed, including: episomal vectors, sendai virus, mRNA, miRNA, adenovirus, transposons, recombinant proteins, and minicircles (Fusaki, Ban, Nishiyama, Saeki, & Hasegawa, 2009; F. Jia et al., 2010; D. Kim et al., 2009; S. L. Lin et al., 2008; Miyoshi et al., 2011; Okita et al., 2011; Stadtfeld, Nagaya, Utikal, Weir, & Hochedlinger, 2008; Warren et al., 2010; Woltjen et al., 2009; Yu et al., 2009). These new methods allow transient expression of the Yamanaka factors in the first few passages of the cells without the need for integration into the host genome, thus mitigating any risk of aberrant gene expression by random integration of the factors into an active transcription or enhancer site. With this
new technology, researchers have begun to utilize iPSCs to study development, as well as, disease mechanisms (Shi, Inoue, Wu, & Yamanaka, 2017; K. Takahashi & Yamanaka, 2016).

1.1.4 iPSC-based models of complex, neurological disorders

Complex neuropsychiatric and neurocognitive disorders may be considered distinctly human diseases. Human postmortem samples only provide a snapshot of the disease, which often progresses for decades, making interpretations difficult and mechanistic studies impossible with these samples alone. Animal models have helped reveal many important mechanisms for various neurological disorders, but there are limitations. While there is high conservation between mice and other animal models and humans for brain development and function, the human brain is a much more evolved structure with unique characteristics. These differences present themselves in development, cell morphology and function, and disease progression (Hodge et al., 2019; J. A. Miller, Horvath, & Geschwind, 2010). For instance, human astrocytes are on average 2-3 times larger and have up to 10-fold more processes than rodent counterparts. Human protoplasmic astrocytes also have faster activity with the ability to propagate calcium four time faster than rodents, and there are several more subtypes of human astrocytes than rodent (Oberheim et al., 2009). Human astrocytes are also significantly different at the gene expression level. In one study, human astrocytes had 655 astrocyte enriched genes that were at least four-fold higher expressed than mice, and conversely,
mice had 259 astrocyte enriched genes that had at least four-fold increased expression over human astrocytes (Y. Zhang et al., 2016). Similar differences were found in microglia (Gosselin et al., 2017). In sum, the rodent counterparts to human cells are much smaller, less complex, and have significantly different transcriptomes, leading to complications in modeling human development in animals.

Differences between humans and mice during disease have also been described. Mouse models of Alzheimer’s disease have consistent issues of developing both plaques and tangles seen in humans with Alzheimer’s. In fact, it requires inserting three mutated genes, APP$_{\text{Swe}}$, Tau$_{P301L}$, and PS1$_{M146V}$, to develop plaques, tangles, and the functional deficits in mice. The APP or PS1 mutation alone in humans would produce all three pathologies (Oddo et al., 2003). Transcriptomic analysis of whole brains have also shown enrichment of disease genes, especially dementia-associated genes, that are enriched in humans but not mice (J. A. Miller et al., 2010). Microglia specific transcriptomic analysis has also revealed significant differences between humans and mice in Alzheimer’s and Parkinson’s related genes (Gosselin et al., 2017). These genetic and functional differences between species may help explain why so many therapeutics that have been successful in animal models fail clinical trials. This further exposes the need to utilize more human-specific models to better understand diseases.

iPSCs have been used to model a wide variety of neurological disorders including familial and sporadic Alzheimer’s, familial and sporadic Parkinson’s,
familial dysautonomia, motor neuron disease, amyotrophic lateral sclerosis (ALS), Schizophrenia, and fragile X syndrome, as well as, perform drug screens targeting several of these diseases (Aflaki et al., 2016; Brennand et al., 2015; Hoing et al., 2012; Kaufmann et al., 2015; Lee et al., 2009; Nagai et al., 2007; H. N. Nguyen et al., 2011; Yagi et al., 2011; Young et al., 2015). Co-culture with multiple disease relevant cell types has further enhanced the ability to recapitulate diseases in vitro. For example, primary astrocytes with a disease-relevant mutation in the \textit{SOD1} gene co-cultured with mouse ESC-derived motor neurons or GABAergic interneurons has been used to study ALS (Nagai et al., 2007). This modeling platform recapitulated cell-autonomous and non-cell-autonomous factors contributing to ALS development and showed that astrocytes with mutated \textit{SOD1} released factors which were selectively toxic to motor neurons and not GABAergic interneurons. Several other groups utilized similar co-culture systems of primary and mouse and human ESC-derived cells and had similar findings on the role of astrocytes in ALS (Di Giorgio, Carrasco, Siao, Maniatis, & Eggan, 2007; Haidet-Phillips et al., 2011; Marchetto et al., 2008). In addition to ALS, co-cultures have been used to model Alzheimer’s disease with a tri-culture of HiPSC-derived neural progenitors, astrocytes derived from a human neural progenitor cell line, and the immortalized human microglia SV40 cell line (Park et al., 2018). Lastly, co-cultures consisting of multiple HiPSC-derived cell types have been reported (Haenseler et al., 2017). These advanced cultures better replicate the intricate interactions that occur between cell types during disease development than mono-cultures, especially in complex, neurological
disorders that take years to decades to develop. 2D iPSC cultures, using one or multiple cell types, have already provided a wealth of knowledge on the development and mechanisms of neurological disorders.

In addition to 2D cultures, HiPSCs can be differentiated into 3D organoids. Organoids have the unique ability to establish connections among cells through a much more physiologically representative structure. Organoids have been used to model several organs including the small intestine, liver, pancreas, and brain among others (Rossi, Manfrin, & Lutolf, 2018). Brain organoids can grossly recapitulate the cortical lamination and represent early-mid gestation fetal brain development. These organoids have been used to model several neurological disorders including, microcephaly, autism, and Zika infection (Kelava & Lancaster, 2016). In fact, organoids have uncovered several potential mechanisms to these disorders. This includes a finding that organoids from individuals with autism overproduce GABAergic interneurons due to overexpression of FOXG1 (Mariani et al., 2015). However, since it takes 50 or more days for astrocytes to appear in the organoids (Pasca et al., 2015), gene expression is similar to early fetal brain, and extrinsically generated cell types, such as microglia and vasculature, would need to be added separately, there are still caveats to interpreting the data and applying it to diseases that take decades to develop. Regardless, organoids are a promising advancement in iPSC technology that will allow researchers to ask questions that could not be asked in 2D cultures.
iPSC cultures of various complexities have been utilized to study neurological disorders both at the mechanistic and therapeutic levels. As differentiations and culture systems become more representative of their in vivo counterparts, these systems will become even more useful. This is especially necessary for the most complex and broad disorders that are caused by a multitude of factors including environmental factors and potentially hundreds of genes such as schizophrenia, and disorders like HIV-associated neurocognitive disorders that are caused by a virus that exclusively infects humans.

1.2 Schizophrenia

1.2.1 Etiology and symptoms of schizophrenia

Schizophrenia (SZ) is a heterogeneous disorder generally characterized by adolescent onset of psychosis together with deteriorations of social and cognitive functioning (Carpenter & Buchanan, 1994; Lewis, 2012). Symptoms can be broken into three major groups, positive, negative, and cognitive symptoms. Positive symptoms include hallucinations and delusions; whereas, negative symptoms include depression, loss of initiative, anhedonia, and social withdrawal. Cognitive symptoms include attention deficits, impaired working memory and verbal learning, and impaired executive functions among others. It occurs in roughly 1% of the population and treatments with first- and second-generation antipsychotic agents and nonpharmacological therapies have helped reduce relapse from 60%-80% to 18%-32%. (Carpenter & Buchanan, 1994; Patel, Cherian, Gohil, & Atkinson, 2014). However, the mechanisms are not well understood and the etiology is multi-factorial (Patel et al., 2014), contributing to
poor advancement in therapeutics. iPSC cultures that can replicate the pathology may help further elucidate these mechanisms.

Observable pathologic changes in brains of SZ patients are varied. (Bowie & Harvey, 2006; Kahn et al., 2015). There are findings that show overall decreases in the number of excitatory synapses, although non-uniform across regions (Osimo, Beck, Reis Marques, & Howes, 2019) and impaired glutamatergic neuron dendrite morphology in SZ patients (Hu, MacDonald, Elswick, & Sweet, 2015). In addition, GABAergic neuron activity and levels of GAD1, the enzyme necessary for GABA synthesis, is downregulated (Inan, Petros, & Anderson, 2013; Kahn et al., 2015). Lastly, dopaminergic neurons may also play a complicated role in the pathophysiology (Seshadri, Zeledon, & Sawa, 2013). Taken together, these findings suggest multiple neuron subtypes are involved in the pathology of SZ consistent with the disorder being defined by a constellation of symptoms, rather than a specific genetic or brain abnormality.

Inflammation has been implicated in the waxing and waning of symptoms in SZ patients. Patients have exhibited increased levels of IL-6, IL-1β, IFNγ, and other inflammatory cytokines in blood and cerebral spinal fluid (CSF) (B. J. Miller, Buckley, Seabolt, Mellor, & Kirkpatrick, 2011; Potvin et al., 2008). However, how these cytokine changes are linked to changes in the brain parenchyma remain unknown. Many factors including smoking, stress, BMI, co-morbidities, and inconsistent patient follow ups contribute to data variability that hamper clear interpretation of these clinical findings. Studies have also investigated inflammation by microglia, the resident immune cell of the CNS, by
in vivo and in vitro methods. There have been mixed findings on activated microglia in postmortem studies (Fineberg & Ellman, 2013). In addition, several in vitro studies have suggested microglia increase synaptic phagocytosis in SZ (Sekar et al., 2016; Sellgren et al., 2019; Sellgren et al., 2017). Overall, there is a large body of literature, although variable in its findings and level of scrutiny, that suggests a role for inflammation and microglia activation in SZ.

Metabolic compromise, including mitochondrial dysfunction, has long been posited to contribute to the development of symptoms in SZ (Konradi & Ongur, 2017; Whatley, Curti, & Marchbanks, 1996). Recent studies have further strengthened this notion. For example, gene expression alterations in postmortem neocortical pyramidal neurons suggest disruption of mitochondrial function (Arion et al., 2017), and other studies reported mitochondrial defects in neural progenitors and cortical interneurons (cINs) (Ni et al., 2019) derived from iPSCs from patients with SZ relative to healthy controls (Brennand et al., 2015). However, despite the potential importance to the discovery of novel treatments for SZ, evidence for mitochondrial dysfunction in SZ in living, human neurons with a defined genetic alteration has been lacking.
1.2.2 22q11.2 Deletion Syndrome

Figure 3: 22q11.2 region with select, associated genes

Within the 22q11.2 region, there is roughly 40 genes. *MRPL40* functions in the translation of mitochondria-encoded genes, and *CLDN5* produces a tight junction protein found in the endothelial cells of the blood brain barrier. Adapted from (Karayiorgou, Simon, & Gogos, 2010).

22q11.2 deletion syndrome (22qDS, also known as DiGeorge’s syndrome, velo-cardio-facial syndrome) is a genetic condition due to a 3 megabase hemizygous deletion of approximately 40 genes on chromosome 22 (Figure 3) (Gur et al., 2017). These patients present with cardiac malformations, facial deformities, gastrointestinal disturbances, and intellectual disability, as well as, increased rates of neuropsychiatric conditions including attention deficits, autism spectrum disorder, and SZ (Gur et al., 2017). The 22qDS deletion includes gap junction and mitochondria related genes influencing blood brain barrier function (Arinami, 2006; Devaraju & Zakharenko, 2017; Greene, Hanley, & Campbell,
2019; Greene et al., 2018), however the status of the blood brain barrier (BBB) in 22qDS is unknown.

The most prevalent genetic risk factor for SZ is 22qDS, occurring in about 1:4000 births (Scambler, 2000). Roughly one-quarter of people with 22qDS develop SZ, a 25-fold increase from the general population, in a manner that is not grossly distinguishable from non-syndromic SZ (Chow, Watson, Young, & Bassett, 2006; D. Sun et al., 2018). Remarkably, in 22qDS six of the approximately 45 deleted genes encode for proteins that are mitochondrial-localizing, and three others strongly influence mitochondrial function (Maynard et al., 2008; Napoli et al., 2015). One study has found evidence for mitochondrial dysfunction in serum samples from subjects with the 22qDS (Napoli et al., 2015), while another recent study used proteomic analyses in fibroblasts from 22qDS subjects together with analyses of 22q model transgenic mice and flies to conclude that mitochondrial SLC25A1/A4 and TXNRD2 influence synaptic function (Gokhale et al., 2019). The importance of TXNRD2 in long range cortical connectivity and psychosis-related cognitive deficits was further established recently in transgenic mice (Fernandez et al., 2019). Various models have implicated mitochondria-related deficits in schizophrenia development in the context of 22qDS.

1.2.3 MRPL40

Mitochondrial ribosomal protein L40 (MRPL40) is a nuclear encoded gene in the 22q11.2 locus that produces a protein that localizes to the mitochondria
and assists in translation of mitochondria encoded genes (Kenmochi et al., 2001; Maynard et al., 2008). The mitochondria genes encode subunits for several of the complexes in the electron transport chain, specifically Complex I, II, IV, and V (Wallace, 1992). \textit{MRPL40} was identified as a candidate SZ risk gene (Carrera et al., 2012), and transgenic mice lacking one copy of \textit{MRPL40} show alterations in mitochondrial calcium as well as psychosis-related cognitive deficits (Devaraju et al., 2017). However, to our knowledge, no one has utilized human-specific, living neurons form 22qDS patients with schizophrenia to further investigate the potential mitochondria-related impairments.

\subsection*{1.2.4 Blood Brain Barrier}

The blood brain barrier (BBB) is comprised of endothelial cells, pericytes, and astrocytes and restricts transportation into and out of the brain parenchyma (Abbott, Patabendige, Dolman, Yusof, & Begley, 2010). The vasculature of the BBB is characterized by specialized transport systems, higher mitochondrial volume fraction, and a paracellular cleft between adjacent endothelial membranes (Abbott, 2000; Abbott, Ronnback, & Hansson, 2006; Obermeier, Daneman, & Ransohoff, 2013; Pan et al., 2011). The anatomical basis of barrier function is due to an elaborated network of junctional proteins composed of tight junctions (TJs) and adherens junctions (AJs) (Alvarez et al., 2015; Muldoon et al., 2013; Zihni, Mills, Matter, & Balda, 2016). This network confers immune privilege to the CNS, by restricting interactions with the peripheral immune system (Abbott et al., 2010; Engelhardt & Coisne, 2011; Muldoon et al., 2013).
Additionally, immunoquiescent properties of the BBB endothelium also contribute to CNS immune privilege, specifically by downregulating expression of cell adhesion molecules, cytokines, and chemokines to further impede leukocyte transmigration into the CNS (Alvarez et al., 2015). Interestingly, fluctuations of psychotic symptoms in SZ resemble the relapsing remitting pattern of prototypical immunological disorders, suggesting that the peripheral inflammation observed in SZ patients may contribute to disease progression (Eaton et al., 2006; Endres et al., 2015; Muller, 2018; Severance, Yolken, & Eaton, 2016). In addition, reduced CLDN5 expression, one of the haploinsufficient genes in 22q11DS (Figure 3), has been associated with SZ (Greene et al., 2018). As the BBB stands as the interface between the CNS and the periphery (Abbott et al., 2010), and because clinical data suggests that the BBB is affected in SZ (Aleksovska et al., 2014; Nishiura et al., 2017; Pollak et al., 2018), compromised BBB may contribute to disease progression (Greene et al., 2019; Kealy, Greene, & Campbell, 2018; Pollak et al., 2018). Therefore, the immunoquiescent properties of the CNS vasculature in the context of 22q11DS should be further investigated.

Both barrier function and immune privilege of the BBB are modulated by the influence of astroglial processes ensheathing the CNS vasculature (Abbott et al., 2006; Alvarez, Dodelet-Devillers, et al., 2011; Cheslow & Alvarez, 2016). The close association of these perivascular astrocytes with the CNS vasculature positions them not only as critical cells to regulate CNS blood flow (Koehler,
Roman, & Harder, 2009) and supply glucose to neurons (Verkhratsky & Nedergaard, 2018), but also the first cells to encounter breaches in the BBB (Alvarez, Cayrol, & Prat, 2011; Colombo & Farina, 2016). Astrocytes are key in the inflammatory cascade that results when the relative immune privilege status of the CNS is disrupted, a process termed neuroinflammation (Brambilla, 2019). As part of their role in the innate immune response, astrocytes are known to become activated, producing cytokines and chemokines (Brambilla, 2019; Colombo & Farina, 2016). In light of the renewed emphasis on neuroinflammatory mechanisms in SZ (Miyaoka et al., 2017; Muller, 2018; Pollak et al., 2018; Severance et al., 2016; Wei & Hemmings, 2005) and as glial activation has been reported in the post mortem SZ brain, (Catts, Wong, Fillman, Fung, & Shannon Weickert, 2014; Colombo & Farina, 2016) astrogliosis may play a role in the development of SZ in 22qDS patients.

1.3 HIV

1.3.1 Pathology and treatment of HIV infection

Like the disorder schizophrenia, HIV infection, and the consequential acquired immunodeficiency syndrome (AIDS), is another human specific disease. The first cases of AIDS were reported in 1981 (Gottlieb et al., 1981). Human Immunodeficiency Virus (HIV), the cause of AIDS, was not isolated from T lymphocytes until 1983 (Barre-Sinoussi et al., 1983) and confirmed as the cause of AIDS until 1984 (Brun-Vezinet et al., 1984; Schupbach et al., 1984). Initially called lymphoadenopathy-associated virus (LAV) (Montagnier et al., 1984), HIV is a human-specific retrovirus, part of the human T-cell leukemia virus (HTLV)
family (Barre-Sinoussi et al., 1983). HIV evolved from simian immunodeficiency virus through cross-species transmission (Hirsch, Olmsted, Murphey-Corb, Purcell, & Johnson, 1989; Peeters et al., 1989). Initially thought to be horizontally transmitted through sex or blood transfusion or blood to blood contact (Barre-Sinoussi et al., 1983), HIV can also be transmitted vertically, from mother to baby (Forbes et al., 2012). There are two major types of HIV, HIV-1 and HIV-2. Most infected patients have HIV-1. HIV-2 is a less serious version that has a lower ability to be transmitted, slower progression to AIDS, and higher survival probability without antiretroviral treatment (Esbjornsson et al., 2018; Nyamweya et al., 2013). There are currently 40 million individuals infected worldwide that require constant treatment for the rest of their lives, causing a large strain on healthcare. Using new models including iPSC-derived models, can help develop potential cures or vaccines.
Antiretrovirals have been designed to target nearly every stage of the HIV replication cycle. We focused on inhibiting reverse transcriptase. This allows previously infected cells to continue producing new virions that can enter an uninfected cell but will not be integrated, creating an infected population and an uninfected, activated population. Adapted from (Barre-Sinoussi, Ross, & Delfraissy, 2013).

Once in a host, HIV infects cells via the CD4 receptor using chemokine receptors, CCR5 and/or CXCR4 as a co-receptor (Dalgleish et al., 1984; Feng, Broder, Kennedy, & Berger, 1996; Weiss, 2013) resulting largely in infection of CD4 positive T lymphocytes and macrophages. Initial infection usually targets
cells with high CD4 expression and the CCR5 co-receptor, which are largely T lymphocytes. However, CXCR4-tropic, as well as, CCR5-tropic strains that can infect low expressing CD4 cells, such as macrophages and microglia arise later in disease progression (Joseph, Arrildt, Sturdevant, & Swanstrom, 2015; Weiss, 2013). When HIV binds with the receptors via the viral protein gp120, the viral envelope fuses to the host cell membrane by viral gp41 and releases its capsid into the cell (Weiss, 2013). As the capsid is shuttling to the nucleus, reverse transcriptase converts the single-stranded RNA genome into double-stranded DNA. With the viral protein integrase, the viral DNA is integrated into the host genome. The host machinery will transcribe and translate the HIV genome, at which point it assembles into new virions that are subsequently released from the host cell. In the final stage of the replication cycle, the HIV protease cleaves the Gag and GagPol polyproteins resulting in fully mature virus, which can infect new host cells (Feinberg & Greene, 1992; Freed, 2015).

During initial infection, or the eclipse phase, few symptoms are seen in the patients. However, during this time the viral reservoir is established. In the acute phase, between 3- and 9-weeks post infection, patients begin to experience flu-like symptoms and cytotoxic T lymphocytes respond to the virus as HIV RNA plasma levels hit peak amounts, and CD4+ T cell counts drop to less than 350 cells per µL blood. CD4+ T cell counts rebound slightly by 12 weeks post infection, but then consistently drop in the months and years following in the chronic phase. As T cell counts drop to below 100 cells per µL blood,
opportunistic infections and symptoms, including coccidioidomycosis, progressive multifocal leukoencephalopathy, toxoplasmosis of the brain, and encephalopathy, become much more prevalent, eventually leading to death within 10 years of initial infection. However, with the advent of antiretroviral therapy (ART) and combined antiretroviral therapy (CART), life expectancy is now within a couple years as those who are uninfected (Deeks, Overbaugh, Phillips, & Buchbinder, 2015).

Antiretrovirals have been developed to inhibit nearly every stage of the replication cycle (Figure 4) (Barre-Sinoussi et al., 2013). Antiretrovirals can be broken down into five major classes: nucleoside reverse transcriptase inhibitor (NRTI), non-nucleoside reverse transcriptase inhibitor (NNRTI), integrase strand transfer inhibitor (INSTI), protease inhibitor, and entry inhibitor (Deeks et al., 2015). Currently, the frontline, recommended treatment regimen is one INSTI and two NRTIs (Kulkarni, Hluhanich, McColl, Miller, & White, 2014). In addition, a preexposure prophylaxis (PrEP) with the NRTIs tenofovir and emtrictabine (Saag et al., 2018) has been found to reduce risk of infection in individuals at high risk of HIV exposure such as non-discordant couples and sexually active adolescents. However, each drug has its own side effects, pharmacokinetics, and CNS penetrance. For instance, the NRTI Zidovudine, the NNRTI Efavirenz, the protease inhibitor darunavir, and the INSTI raltegravir have relatively high CNS penetrance (de Oliveira, do Olival, & de Oliveira, 2015). While this is advantageous for targeting HIV infection in the CNS, several of these drugs may
have neurotoxic effects. For example, efavirenz has established short and long
term neurotoxic effects, and the protease inhibitors ritonavir and saquinavir,
individually and in combination with the NRTI zidovudine, leads to dendritic
damage and oxidative stress *in vitro* (Cagla Akay et al., 2014; Ciavatta et al.,
2017; Grilo et al., 2017; Robertson, Liner, & Meeker, 2012). As the toxic effects
of ART in the CNS are not well defined, new models like iPSC-derived cultures,
need to be utilized to further understand the negative effects of chronic ART use.

HIV can enter the CNS within two weeks of initial infection (Davis et al.,
1992; Valcour et al., 2012). HIV enters the brain parenchyma when peripheral,
infected lymphocytes and monocytes transmigrate through the blood brain barrier
(Saylor et al., 2016). Free virions may also directly transmigrate through the
blood brain barrier (Argyris et al., 2003; Bobardt et al., 2004). Although this
mechanism is not well characterized and most likely accounts for a small
percentage of virus entering the parenchyma (Atluri et al., 2015). Once the
monocytes enter the parenchyma, they differentiate into macrophages and
release new virions, leading to infection of microglia, the resident immune cell
and the only cell that can be productively infected in the brain by HIV. Microglia
constitute the HIV reservoir in the brain (Castro-Gonzalez, Colomer-Lluch, &
Serra-Moreno, 2018; Wallet et al., 2019). Activated, uninfected astrocytes and
microglia as well as infected microglia release inflammatory cytokines and
chemokines, excitotoxic factors including glutamate, and reactive oxygen species
(Figure 5). (Garvey et al., 2014; Ghorpade et al., 2005; Saylor et al., 2016; M. L.
Zhao, Kim, Morgello, & Lee, 2001). This activation leads to chronic low level inflammation throughout the individual’s life even with ART (Kolson, 2017), leading to neuronal damage (O’Donnell et al., 2006) and thus neurological disorders.

Figure 5: Proposed mechanisms underlying HAND development

Infected monocytes transmigrate through the blood brain barrier, into the brain parenchyma. At which point, they differentiate into macrophages and release virions that infect the microglia. The infected microglia and macrophages as well as uninfected, activated astrocytes and microglia release excitotoxic factors and inflammatory cytokines. As microglia are the only resident cell in the central nervous system that can be productively infected, there is increased focus on its role in HAND
1.3.2 HIV-Associated Neurocognitive Disorders

HIV-Associated Neurocognitive Disorders (HAND) are a chronic, progressing spectrum of neurologic disorders. HAND can be broken into three major categories of severity. The least severe form is Asymptomatic neurocognitive impairment (ANI) and accounts for roughly 30% of HIV infected individuals on CART. Patients with ANI have impairments in at least two neurocognitive domains that are at least standard deviation from the norm (Saylor et al., 2016). Neurocognitive domains include perceptual-motor function, language, learning and memory, social cognition, complex attention, and executive function (American Psychiatric Association, 2013). While individuals with ANI do not have noticeable impairment in their daily function, they are 2-6 times more likely to develop more severe, symptomatic forms of HAND than neurocognitively normal, HIV-infected individuals (Grant et al., 2014). The second most severe form is Mild neurocognitive disorder (MND), which occurs in 10-15% of HIV infected patients on CART. Individuals have impairment in at least 2 neurocognitive domains by at least one standard deviation from the median, much like ANI, but they also experience moderate impairment in their daily activities. The third and most severe form is HIV-associated dementia (HAD). This form occurs in 2-8% of HIV infected individuals on CART. Patients have impairments in at least two neurocognitive domains by at least two standard deviations from the median. In addition, they experience significant interference in their daily activities, with
symptoms very similar to non-HIV dementia. Before the advent of CART, the more severe forms of HAND, MND and HAD, were more prevalent. However, while CART has reduced the severity of HAND, the prevalence has actually increased due to the increased lifespans of the HIV-infected individuals (Figure 6) (Saylor et al., 2016). Thus, CART by itself is not enough to treat HAND, and additional therapies need to be developed.

The major pathologic manifestation that persists in HAND patients with ART is synaptodendritic damage and the accumulation of microglia, the resident immune cell of the central nervous system (CNS); however, the mechanisms underlying synaptic damage remain elusive. Synapse loss is associated with infiltration of macrophages from outside of the CNS and activation of microglia. Both HIV-infected macrophage populations can release cytokines, viral proteins, and excitotoxins, which can lead to synaptic damage (Saylor et al., 2016) and are potential reservoirs for the virus (Castellano, Prevedel, & Eugenin, 2017). While patients on ART experience milder forms of HAND (Figure 6), they still experience chronic inflammation (Kolson, 2017). Infected microglia and uninfected, but active microglia may be working in tandem to slowly release proinflammatory cytokines and reactive oxygen species that, over time, can lead to synaptodendritic damage (Sui et al., 2007; Turchan–Cholewo et al., 2009). Additional aspects of inflammatory responses have been implicated in HAND, including the integrated stress responses (ISR) and its resultant activation of EIF2 (C. Akay et al., 2012; Lindl, Akay, Wang, White, & Jordan-Sciotto, 2007).
These signaling pathways have not been efficiently studied, especially not on a cell type basis.

**Figure 6: Frequencies of HAND disorders before and during CART era**

Combined antiretroviral therapy (CART) has led to decrease in the frequency of the most severe forms of HAND, HIV Associated Dementia (HAD) and Mild Neurocognitive Impairment (MND). However, the prevalence of HAND stays the same as the before CART era. Adapted from (Saylor et al., 2016).

While the major pathological manifestation of HAND is synaptodendritic damage, the response during initial exposure to HIV and ART is unknown largely because current models for HIV-mediated neuroinflammation are limited by species differences and human tropism of the virus. For instance, the HIV-1 Tat transgenic mouse model exhibits neuroinflammation and behavioral deficits, but only expresses a single viral protein from astrocytes (B. O. Kim et al., 2003). Another HIV-1 transgenic mouse model has neuroinflammation, but is missing
two of the genes, *gag* and *pol*, and is expressed in every cell in the body (Reid et al., 2001). Lastly, while isolated microglia from human patients can provide important insights, this is only a snapshot of the end stage of the disease. Therefore, the development of a novel *in vitro* human system allowing the interaction of the main cell types involved in HAND is needed to further understand the neuropathogenesis and develop novel therapeutics.

### 1.3.3 Microglia

Microglia originate from the first wave of hematopoietic progenitors that arises from the blood islands in the extra-embryonic yolk sac (Ginhoux et al., 2010). These progenitors are RUNX1⁺CSF1R⁺ c-Myb⁻ and can differentiate into erythroblasts, megakaryocytes, and macrophages (Ginhoux & Guilliams, 2016). When these progenitors enter the brain during early development, they differentiate into microglia and become self-sustaining (Ginhoux et al., 2010). Microglia account for 5-20% of all glial cells and are the resident immune cell of the CNS (Ginhoux, Lim, Hoeffel, Low, & Huber, 2013). In addition to immune surveillance, microglia also serve important roles in early development and homeostasis in the brain. For example, they regulate axon wiring of dopaminergic neurons in the forebrain as well as positioning of a selection of neocortical interneurons (Squarzoni et al., 2014). Microglia also prune excess synapses during development in a complement-dependent manner (Schafer et al., 2012; Stevens et al., 2007), promote branching of sprouting blood vessels, release neurotrophic factors including BDNF and IGF-1, and can induce
apoptosis in certain neuronal subsets and subsequently phagocytose and clear the apoptotic neurons during early development (Kierdorf & Prinz, 2017). In adulthood, microglia help regulate activity-dependent synaptic plasticity, neurogenesis, and learning and memory (Salter & Stevens, 2017). While microglia are extraordinarily important during early development and homeostasis, they have also been implicated in multiple neurological disorders.

Activated microglia aberrantly phagocytose synapses in multiple neurological disorders including: Alzheimer’s disease, frontotemporal dementia, and multiple sclerosis (Keren-Shaul et al., 2017; Lui et al., 2016; Salter & Stevens, 2017; Sui et al., 2007; Zrzavy et al., 2017). Beyond phagocytosis, microglia can also release neurotoxic cytokines, which has been shown in Alzheimer’s disease. Lastly, $TREM2$, a gene highly expressed in microglia, has been identified as a risk gene for Alzheimer’s disease (Keren-Shaul et al., 2017), frontal temporal dementia, and Parkinson’s (Krasemann et al., 2017). Although the exact mechanism on how variants in $TREM2$ lead to neuronal damage and pathology in these disease is not well understood (Salter & Stevens, 2017). While patients on ART experience milder forms of HAND, they still experience chronic inflammation (Kolson, 2017). As microglia are implicated in many neurological disorders, are the resident immune cell of the CNS, and are the only cell in the CNS that can be infected by HIV, microglia may play a large role in the development and progression of HAND. However, it is not well understood how microglia may contribute.
1.3.4 Astrocytes

Astrocytes and neurons share a primary progenitor cell, radial glia, (Kriegstein & Alvarez-Buylla, 2009) and are the most abundant glial cell type in the brain (Jakel & Dimou, 2017). They are important in CNS homeostasis, including synapse formation and maturity and regulation of water and ion transportation (Almad & Maragakis, 2018). This includes their role in the tripartite synapse, which is the combined structure of the presynaptic terminal, postsynaptic terminal, and astrocyte. In the tripartite synapse, the astrocyte acts as a modulator of synaptic activity. The astrocytes react to neuronal activity with an influx of Ca$^{2+}$, leading to release of neurotransmitters including glutamate and D-serine, which in turn regulate neuronal activity. The level of regulation is region specific. For instance, the cerebral cortex has heavy coverage of the synapses by the astrocytes, but the CA1 region of the hippocampus has much lower coverage (Araque, Parpura, Sanzgiri, & Haydon, 1999; Santello, Toni, & Volterra, 2019). This complex interaction between the synapse and astrocytes is crucial in several functions including long-term potentiation and synaptic plasticity as well as high order functions such as learning and memory (Almad & Maragakis, 2018; Santello et al., 2019).

While astrocytes have extensive functions in development and homeostasis maintenance, they are also implicated in multiple neurological disorders. Mutations in GFAP leading to a toxic gain of function are implicated in Alexander’s disease. MECP2 deficiencies, which causes Rett syndrome, in astrocytes results in astrogliosis, impaired glutamate clearance, and impaired
cytokine and growth factor production. With astrocytes’ role in metabolism as well as potassium and glutamate regulation, they have been implicated in epilepsy. In fact, knock out of the glutamate transporter $GLT1$ leads to seizures and death. $GLT1$ and $SOD1$ mutations in astrocytes have been implicated in Amyotrophic lateral sclerosis. Astrocytes have also been implicated in Alzheimer’s disease, with increased, reactive astrogliosis around Aβ plaques and increased TNFα production being standard symptoms of the disease (Almad & Maragakis, 2018). Lastly, a subset of neurotoxic astrocytes, deemed A1 astrocytes, have been reported in multiple disorders including Alzheimer’s disease, Amyotrophic lateral sclerosis, and Parkinson’s disease. Astrocytes morph into the A1 state with exposure to C1q, TNF, and IL-1α that are produced from microglia. Once in the neurotoxic, A1 state astrocytes cause neuron and oligodendrocyte death and have impaired phagocytosis and synaptogenesis (Liddelow et al., 2017). Overall, astrocytes play important roles in health and disease.

1.3.5 Integrated Stress Response

The integrated stress response (ISR) is a highly conserved pro-survival signaling pathway that activates in response to a broad range of stimuli, including external factors such as nutrient and oxygen deprivation and exposure to harmful pathogen such as viruses, as well as, internal factors such as endoplasmic reticulum (ER) stress. When triggered, the ISR utilizes one or a combination of four serine/threonine kinases, GCN2 (general control nonderepressible 2), PKR (double-stranded RNA-dependent protein kinase R), HRI (heme-regulated eIF2α
kinase), or PERK (protein kinase R (PKR)-like ER kinase), depending on the stress signal, to phosphorylate eIF2α at Ser51 of the α-subunit. GCN2 is activated in response to amino acid deprivation. PKR responds to viral infection. HRI is activated by heme deprivation. Lastly, PERK responds to ER stress (Figure 7) (Pakos-Zebrucka et al., 2016). All kinases culminate in the phosphorylation and activation of EIF2, leading to global translation inhibition (Bond, Lopez-Lloreda, Gannon, Akay-Espinoza, & Jordan-Sciutto, 2020).

Figure 7: Integrated Stress Response pathway

Four kinases (GCN2, PKR, HRI, and PERK) are activated by different stimuli, but all converge on phosphorylating eIF2α (p-eIF2α). P-eIF2α halts global cap-dependent translation and promotes ATF4 to enter the nucleus and promote gene expression of specific, homeostatic genes. ATF4 also
The eIF2 (eukaryotic translation initiation factor 2) complex consists of eIF2α, eIF2β, and eIF2γ. The complex assists in bringing the methionine-tRNA to the AUG start codon in the ribosome. The subunit eIF2α contains the regulatory phosphorylation site as well as the RNA binding site (Walter & Ron, 2011). Phosphorylated eIF2α inhibits the complex and thus represses global translation of 5’ cap-dependent proteins except for a few homeostatic proteins, including ATF4 (activating transcription factor 4), and activates NF-κB by repressing production of IκB, the inhibitor of NF-κB (Janssens, Pulendran, & Lambrecht, 2014). ATF4 induces transcription of several genes including the pro-apoptotic factor CHOP, GADD34 which negatively regulates eIF2α by de-phosphorylation, and several genes necessary for amino acid metabolism and redox control. The overarching goal of the ISR is to return the cell to homeostasis, and if it cannot, it will induce apoptosis (Bond et al., 2020; Janssens et al., 2014; Pakos-Zebrucka et al., 2016; Walter & Ron, 2011).

1.3.6 Unfolded Protein Response

One part of the ISR is the unfolded protein response (UPR). The UPR has three arms: PERK, IRE-1 (inositol-requiring enzyme-1), and ATF6 (activating transcription factor 6). All three arms are transmembrane receptors positioned in between the ER lumen and the cytoplasm and are bound to the chaperone protein BiP during homeostasis. When the UPR sense stress through unfolded
proteins or other stressors, BiP is released from the three arms. PERK and IRE-1
dimerize and trans-autophosphorylate to become active. PERK activation, as
mentioned prior, phosphorylates eIF2α which leads to global translation block
and activation of ATF4. Activated IRE-1 cleaves XBP-1s, which transports to the
nucleus where it promotes gene expression for chaperones and lipid synthesis
as well as ER-associated degradation (ERAD) genes which degrade the
unfolded proteins. Whereas, ATF6 translocates to the golgi apparatus where it is
processed before transporting to the nucleus to also promote gene expression
for chaperones and lipid synthesis that assist in protein folding (Figure 8)
(Janssens et al., 2014). These three arms work in tandem to block new protein
translation, degrade unfolded proteins, and return the cell back to homeostasis.
Figure 8: Unfolded Protein Response pathway

The three arms of the UPR (IRE-1, PERK, and ATF6) sense unfolded proteins in the ER lumen. Once activated, each arm promotes specific gene expression. IRE-1 promotes ERAD genes for degradation through XBP-1. PERK inhibits global translation and promotes homeostatic and redox genes through ATF4. ATF6 promotes chaperone genes for folding proteins. Adapted from (Janssens et al., 2014).

If the UPR is not inhibited once the cells return to homeostasis, it can lead to cell damage and death that can become pathological. The UPR has been implicated in multiple neurodegeneration disorders (Walter & Ron, 2011), including progressive supranuclear palsy, Alzheimer’s disease, Parkinson’s disease, Huntington’s disease, and ALS, among others (Scheper & Hoozemans,
ISR and UPR activation also plays a role in HAND. ATF6β protein levels were increased in the astrocytes and ATF6β and p-eIF2α protein levels in the neurons from postmortem cortical sections from patients with HAND (C. Akay et al., 2012). BiP, the master regulator of the three arms of the UPR, is also increased in HAND patients (Lindl, Akay, Wang, White, & Jordan-Sciutto, 2007). By repressing translation for too long, critical proteins such as synaptic proteins in neurons are lost. In addition, BACE1, which is important in Aβ formation, mRNA translation is increased during PERK activation. Lastly, the UPR can also phosphorylate tau, leading to various tauopathies (Scheper & Hoozemans, 2015). Prolonged activation of the UPR contributes to several neurological disorders through multiple mechanisms, including cell autonomous damage through plaque formation and apoptosis and cell non-autonomous damage through increased inflammation.

1.4 Dissertation overview

By utilizing HiPSCs, we can study disease relevant cell types in a tractable, reductive system. These systems allow us to discover mechanisms of early and late stage disease progression that otherwise would not be possible in postmortem or animal studies. Therefore, we have derived HiPSCs into neurons and endothelial cells with schizophrenia-associated genetic mutations to discover deficiencies in mitochondrial function in excitatory, glutamatergic neurons and impaired barrier function in endothelial cells that are part of the blood brain barrier. In addition, we have created a tri-culture of HiPSC-derived neurons, astrocytes, and microglia to elucidate the reactions and cell-cell interactions at
the transcriptomic and functional level during early HIV infection in the brain. Overall, these studies advance the effort to develop new systems to understand the cellular and molecular mechanisms of human disease and to test novel treatments.
2. Mitochondrial deficits in human iPSC-derived neurons from patients with 22q11.2 deletion syndrome and schizophrenia

2.1 Introduction
Here, we have studied mitochondrial function in iPSC-derived neurons from patients with 22q11DS+SZ (22qSZ) versus healthy controls. Patient-derived neurons have reduced ATP levels, and reduced activity of complexes I and IV of the electron transport chain (ETC). The levels of multiple mitochondrial-translated proteins are reduced, in contrast to the levels of several nuclear-encoded mitochondrial proteins. These findings were replicated in an iPSCs line that we edited to be heterozygous for MRPL40. These results raise suggest that defects in mitochondrial ATP production secondary to reduced levels of mitochondrial-encoded proteins may contribute to neuronal dysfunction in the 22qSZ.

2.2 METHODS
2.2.1 Human induced pluripotent stem cells
Human induced pluripotent stem cells (iPSCs) were contributed by Herbert Lachman (Albert Einstein University). All of the lines from patients with 22q11.2 deletion syndrome were also diagnosed with schizophrenia (D. Zhao et al., 2015). All cell lines had SALSA MLPA KIT P250-A1 DiGeorge (MRC Holland, Amsterdam, Netherlands) testing for the 22q11.2 deletion (Vorstman et al., 2006), and the 22q11.2 deletion lines were confirmed to be hemizygous for the 3mb deletion located in "A-D" region. In addition, each line was tested monthly
and confirmed to be free of mycoplasma. Human iPSCs from 4 lines of affected subjects and 5 lines of controls were applied in this study.

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<th>Cell Line</th>
<th>Age</th>
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</tr>
</tbody>
</table>

**2.2.2 Lentiviral vector generation**

Plasmid of VSVG.HIV.SIN.cPPT.CMV.mNgn2.WPRE and VSVG.HIV.SIN. cPPT.CMV.rTTA. WPRE were kindly provided by Dr. Marius Wernig (Stanford University) and packaged into virus by the University of Pennsylvania Viral Vector Core (VSVG.HIV.SIN.cPPT. CMV.mNgn2. WPRE and VSVG.HIV.SIN. cPPT.CMV. rTTA.WPRE.)
2.2.3 Neuronal differentiation of iPSCs
Induced differentiation of human iPSC lines to neurons (iNrns) was accomplished by an established protocol (Yingsha Zhang et al., 2013). In brief, iPSC lines were infected with two lentiviral vectors: TetOmNgn2-T2A-PuroR and Ubiq-rTTA. On day 1, differentiation was initiated with exposure to doxycycline (2μg/mL, Sigma), followed by puromycin (5μg/mL, Sigma) selection for cells that possessed these two lentiviral vectors 24 hours later. The cells were re-plated the next day and grown in Neurobasal-A medium (Gibco) with B27 (Life Technologies), glutamax (Life Technologies), 5mM glucose, 10mM sodium pyruvate, 10ng/mL NT-3 (Peprotech), 10ng/mL BDNF (Peprotech), and 2μg/mL doxycycline. Rat glia, if used, were added on day 5, and a single administration of 2μM Ara-C (Sigma) was added on day 7. Doxycycline was discontinued on day 10. iNrns were cultured until at least day 21.

2.2.4 Rat glia
Isolated E17 Sprague Dawley rat cortex was obtained from the UPenn NRU Core. Cells were plated onto uncoated 10cm dishes at 1.5million cells/mL. Cells were grown in NM-15 media (Eagle’s MEM with Earle’s salts and 2 mM L-glutamine, 15 % heat-inactivated fetal bovine serum, 6 mg/ml glucose, 0.5 U/ml penicillin, and 0.5 μg/ml streptomycin). Medium exchanges occurred every 5 days, and rat glia grew for at least two weeks. Prior to glial harvest, the glia were shaken at 37°C for 3 hours at 230 rpm to remove microglia. The glia were then dissociated with accutase (Life Technologies), spun down for 5min at 1000 rpm,
resuspended in neurobasal/B27 media, and plated onto the neurons at 100k cells/well of a 24-well plate.

2.2.5 Western blot
Cells were washed with ice-cold PBS twice. Ice-cold RIPA buffer and protease inhibitor cocktail were added next, and cells were scraped from the plate and put into a 1.5mL Eppendorf tube. Cells were placed on ice for 30 min, then centrifuged at 14,000 RPM for 15min at 4 °C. Supernatant was collected and frozen at -80 °C until needed. Protein concentration was quantified with the Pierce BCA kit and a NanoDrop2000 (Thermo scientific) to measure. Samples were loaded on a 4-12% bis-tris gel with LDS Sample Buffer (Life technologies) and Sample Reducing Agent (Life technologies). The SDS-PAGE was run with MOPS buffer (Life Technologies), protein was transferred from gel to nitrocellulose membrane, which was then blocked for 1hr at room temperature using 5% BSA. Li-Cor/Odyssey and Image J are used for data collection and analysis. We used the following primary antibodies: anti-MRPL40 (1:500; Novus), anti-VDAC (1:1,000; Neuromab), anti-Cytochrome b (1:200; Santa Cruz Biotechnology), anti-MT-ND1 (1:500; Abcam), anti-OXPHOS cocktail (1:250; abcam), and β-actin (1:10,000; Cell Signaling Technology). The following Licor secondary antibodies were used all at 1:10,000: IRDye 680LT Goat anti-Mouse, IRDye 680RD Donkey anti-Rabbit, IRDye 800CW Donkey anti-Rabbit, IRDye 800CW Donkey anti-Goat, IRDye 800CW Donkey anti-Mouse.
2.2.6 RNA extraction, reverse transcription, and quantitative PCR
Total RNA was extracted using Trizol (Ambion) and the concentration was measured using nanoDrop2000 spectrophotometer. cDNA was generated using transcript IV VILO Master Mix (ThermoFisher). RNA abundance was measured by Quantitative PCR using TaqMan Gene Expression Master Mix (Applied Biosystems) for primer pairs of ACTIN, MRPL40, VDAC1 and COX1 purchased from ThermoFisher Scientific, and SYBR Green PCR Mix (Applied Biosystems) for primer pairs of Cytochrome b and mt-ND1. Primer sequences: ACTIN (Assay ID: Hs01060665_g1), MRPL40 (Assay ID: Hs00186843_m1), VDAC1 (Assay ID: Hs01019083_m1), COX1 (Assay ID: Hs02596864_g1); Cytochrome b: forward primer: 5′-AGTCCCACCCTCACACGATTCTTT-3′, reverse primer: 5′-AGTAAGCCGAGGGCGTCTTTGATT-3′; mt-ND1: forward primer: 5′-ATGGCCAACCTCCTACTCCTCATT-3′, reverse primer: 5′-TTATGGCGTCAGCGAAGGGTTGTA-3′.

2.2.7 Synapse Counting
Induced neurons grown to day 21 with rat glia were fixed in 4% PFA, stained with DAPI (1:2,000; Invitrogen), VGLUT1 (1:1,000; Sigma), PSD95 (1:500; Neuromab), and MAP2 (1:500; Abcam). The respective secondary antibodies were Donkey Anti-Rabbit Alexa 488 (1:500; Thermofisher Scientific), Goat anti-Mouse Alexa 568 (1:500; Thermofisher Scientific), and Goat anti-Chicken 680 (1:500; Thermofisher Scientific). 40x images were then collected using a Leica DMI8 confocal
microscope. At least 3 images were collected from 3 control and 3 schizophrenic lines of cells. Each neuron imaged had no less than 2 major processes. After collection, images were deconvolved using Hyguens Essential software. Next, images were transferred to Imaris software, where they were then cropped and surfaced by MAP2 staining. PSD95 puncta about 0.5μm in diameter were identified within the MAP2 positive dendrites, then VGLUT1 puncta of 0.5μm localized within 1μm of the PSD-95 were identified. These colocalized puncta within 40μm of the cell soma were quantified as synapses. Cells were counted to normalize for culture density by selecting random areas and counting number of DAPI+MAP2+ cells per unit area.

2.2.8 Preparation of cell lysate for mitochondrial OXPHOS activity
The preparation of cell lysate was conducted as published (Spinazzi, Casarin, Pertegato, Salviati, & Angelini, 2012). Briefly, induced neurons at day 21 were washed with cold PBS, then suspended and centrifuged at 1000rpm for 5 min. Cell pellets were stored at -80 °C until measurement of OXPHOS activity.

2.2.9 Measurement of mitochondria OXPHOS activity and ATP levels
Biochemical methods were used as described (Birch-Machin & Turnbull, 2001). Briefly, cell pellets were thawed, then flash frozen in liquid nitrogen 3 times. Cell lysates were placed into a cuvette with reaction buffer and recorded at the relevant wavelength for each complex assay. Potassium buffer was used in all enzyme activity assays and contains 50mM KCl, 10mM Tris-HCl, and 1mM EDTA with pH 7.4. Reagents for detection of complex I enzyme activity
(wavelength 340nm) include 5mM MgCl2, 2mM KCN, 0.13mM fresh NADH, 65μM CoQ1, 2μg/ml Antimycin, and 2μg/ml rotenone. Reagents for complex II activity (wavelength 600nm) consist of 5mM MgCl2, 20mM succinate, 2mM KCN, 65μM CoQ2, 2μg/ml antimycin, 2μg/ml rotenone, and 50μM Dichlorophenolindophenol. Reagents used for complex III activity (wavelength 550nm) include 5mM MgCl2, 2mM KCN, 15mM fresh cytochrome C, 65μM CoQ2, 0.6mM Dodecyl-β-D-maltoside, and 2μg/ml rotenone. The reagent used for complex IV activity (wavelength 550nm) is 15μM cytochrome c. Complex V (ATP synthase) enzyme activity Microplate Assay Kit (Abcam, ab109714) was applied following manufacturer’s instructions. All chemicals for enzyme analyses were purchased from Sigma-Aldrich. ATP levels were quantified on a Luminescence plate reader using an ATP Bioluminescence Assay Kit (Abcam) following the manufacturer’s instructions. Briefly, cells were lysed by detergent provided in the kit, followed by addition of reconstituted substrate solution and measurement of luminescence. All procedures were performed in the dark.

2.2.10 Generation of MRPL40 heterozygous line
The MRPL40 heterozygous line was generated as described (Ran et al., 2013). Guide RNAs were designed using (http://crispr.mit.edu) to locate to exon 2 of MRPL40. Guide RNAs were ligated into Fast BbsI digested pSpCas9 (BB)-GFP vectors (Addgene, plasmid ID: 48138). The cloned gRNA-Cas9n vectors were introduced into human iPSCs by electroporation under program B16. Human stem cell Nucleofector Kit1 (Lonza) was used. SURVEYOR assays
(Transgenomic, cat.no. 706025), DNA sequencing, and western blot were used to validate the MRPL40 heterozygous mutation. Sequence of gRNAs:

gRNA-Top: 5’-CACCGAAGACAAACAATGACGCTCGC-3’;
gRNABottom: 5’AAACGCGAGC GTC ATTGTTGTCTTC-3’.

MRPL40 primers:

Forward primer: 5’-CCTTCCACGTGACCTTGCT-3’;
Reverse primer: 5’-CCTTCCACGTGACCTTGCT-3’.

2.2.11 Mitochondrial DNA copy number
All reactions were performed in fast optical 96-well reaction plates with barcodes (Applied Biosystems) on SDS7900HT system (CHOP NAPCore). Each sample was analyzed in triplicate. The reaction solution contains 2μl DNA template (3ng/μl), 2μl primers (5μm), 12.5μl SYBR Green PCR Master Mix (Applied biosystems) and 8.5μl H2O. The procedure of amplification program was as follows: 10 min at 95°C, 40 cycles of 15s at 95°C, and 60s at 60°C and melting curve. Relative mtDNA copy number (mtDNA amount/nDNA amount) was calculated by a comparative Ct method, using the following equation:

\[ \text{mtDNA/nDNA} = 2^{-\Delta Ct} \]

Nuclear primers include:

LPL-F; 5’-CGAGTCGTCTTTCTCCTGATGAT-3’,
LPL-R: 5’-TTCTGGATTCCAATGCTTCGA-3’,
B2-microglobulin-F: 5’-TGCTGTCTCCATGTTTGATGTATCT-3’ and
B2-microglobulin-R: 5’-TCTCT GCTCCCACCTCTAAGT-3’.
Mitochondria primers include:

- tRNA Leu(UUR)-F: 5’-CACCCAAGAACAGGGTTTGT-3’,
- tRNA Leu(UUR)-R: 5’-TGGCCA TGGGTATGTGTGA-3’,
- ND1-F: 5’-CCCTAAAACCCGCCACATCT-3’,
- ND1-R: 5’-GCGAT GGTGAGAGCTAAGGT-3’,
- ND4-F: 5’-CCATTCTCTCCTATCCTCAAC-3’,
- ND4-R: 5’-CC ATTCTCCTCCTATCCCTCAC-3’,
- cytochrome b-F: 5’-CACGATTCTTTACCTTTAACCTTTCA TC-3’, and
- cytochrome b-R: 5’- TGATCCCGTTTCGTGCAAG-3’.

### 2.2.12 Electrophysiology

Whole-cell recordings were obtained from neurons with patch pipettes pulled from borosilicate glass (outer diameter, 1.5 mm; inner diameter, 0.86 mm) pulled on a horizontal puller (P-97, Sutter Instruments) and filled with intracellular solution that contained, in mM: K-glutamate, 130; KCl, 6.3; EGTA, 0.5; MgCl2, 1.0; HEPES, 10; Mg-ATP, 4.0; Na-GTP, 0.3. pH was adjusted to 7.30 with KOH; osmolality was adjusted to 285 mOsm with 30% sucrose. When filled with internal solution, pipettes had a resistance of 5-7 MΩ. Calculated chloride equilibrium potential was -73 mV. Unless otherwise specified, chemicals were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Recordings were performed with a MultiClamp 700B amplifier (Molecular Devices, Sunnyvale, CA) using pCLAMP 10 software. Pipette capacitance and series resistance compensation (bridge balance) were applied throughout current-clamp
experiments, with minor bridge balance re-adjustments allowed as required. Signals were low-pass filtered at 10 kHz and sampled at 20-50 kHz and digitized using a Digi data 1550A 16-bit D/A converter (Molecular Devices). Reported values for membrane potential (Vm) and action potential threshold are not corrected for the liquid junction potential. Spontaneous resting membrane potential (Vm) was determined 2 minutes after break-in as the average membrane potential during a 1 second sweep with no current injection. For cells that were spontaneously active, this was measured in the inter-spike interval. Input resistance (Rm) was calculated as the slope of the linear fit to the plot of the V-I relation derived from small subthreshold current steps at/around resting membrane potential. Action potential threshold was calculated as the voltage at which the first derivative (dV/dt) of the AP waveform reached 10mV/ms. Action potential half-width (AP ½-width) is defined as the width of the AP (in ms) at half-maximal amplitude, calculated using AP threshold and the peak of the AP. Action potential after-hyperpolarization (AHP) amplitude is calculated as the depth of the after discharge potential (in mV) relative to AP threshold. Maximal steady-state firing frequency is the maximal mean firing frequency in response to a current injection at which there are no AP failures, with spikes defined as having a clear AP threshold as per above, amplitude of 40 mV or higher, and overshooting -10 mV. Maximal instantaneous firing frequency is the inverse of the smallest inter-spike interval at maximal current step injection. Cutoff frequency (Hz) is defined as the frequency after which a cell can no longer produce action potentials (i.e., there are action
potential failures) in response to repetitive stimulation for 10 seconds.

Experimental design: Data from 7-10 cells per line were included in the analysis.
2.3 RESULTS

(A) Table showing subject characteristics.

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(B) Lentiviral constructs and timeline used to induce neuronal differentiation. Doxycycline is discontinued at 10 days in vitro (DIV).

(C) Epifluorescence image of a DIV21 culture, from a 22qSZ line, with fluorescence immunodetection of the neuronal dendritic marker MAP2. Scale bar: 50 µm

(D) Confocal image processed by deconvolution and Imaris software showing apposition of MAP2 (Blue) with the excitatory presynaptic protein vGLUT (Green), and the excitatory postsynaptic protein PSD95 (Red). Scale bar: 30 µm

(Ryan SK, Li J, Cook K)

2.3.1 HiPSC-derived neurons (iNrs) from subjects with 22q11.2 deletion syndrome and SZ have reduced ATP levels and ETC activity
To investigate the hypothesis that the pathobiology of SZ in the context of 22q11DS could include mitochondrial dysfunction in neurons, we studied 5 iPSC lines from healthy controls and 4 lines from subjects with 22q11.2 deletion syndrome and SZ (22qSZ) that have been published previously (M. Lin et al., 2016) (Figure 9A). These lines were differentiated via the induced expression of neurogenin 2 (Figure 9B), a well-established protocol that rapidly and reproducibly generates a relatively homogenous population of excitatory projection neuron-like cells (Figure 9C and 9D) (Yingsha Zhang et al., 2013).

**Figure 10:** iNrms from subjects with 22qSZ have reduced ATP levels, and reduced ETC complex I and IV activity

(A) ATP level measured by luminescence detection assay at differentiation day (DD) 21. Relative to controls, the 22q11DS group had a nearly 50% reduction of ATP level. Error bars represent SEM.
Mitochondrial complex I (NADH-ubiquinone oxidoreductase; B) complex IV (cytochrome C oxidase; C), complex II (Succinate-ubiquinone oxidoreductase; D) and complex III (Ubiquinone-cytochrome c oxidoreductase; E) was measured in DD21 iPSC-derived neurons. Relative to controls, the 22qSZ group had significant reduction of complex I and IV enzyme activity, but not of complex II or III. n=5 for control, n=4 for 22q11SZ * indicates p<0.05, NS-not significant. Error bars represent SEM. (Ryan SK, Li J)

We first examined ATP production, finding a roughly 50% reduction in the 22qSZ neurons (Figure 10A). To explore potential causes of this decrease, we next investigated enzymatic activity in the electron transport chain (ETC). Both complexes I and IV exhibited significantly reduced activity in 22qSZ neurons (Figure 10B and 10C); however, complex II, III and V (ATP synthase) enzyme activity were unchanged (Figure 10D, 10E and Figure 11A). While all 13 of the mitochondrial DNA-encoded proteins function in the ETC, they are not evenly distributed (Wallace, 1992). The two ETC complexes with significant reductions of activity in the 22qSZ group, complexes I and IV, have seven and three mitochondrial-encoded proteins respectively. In contrast, complexes II, III and V have zero, one and two of these proteins, respectively. The correlation of complex activity reductions to their number of mitochondrial-encoded proteins raises the possibility that an abnormality in mitochondrial translation could be influencing these phenotypes (Boczonadi & Horvath, 2014). That possibility was already implicated by the fact that one of the genes deleted in the 22q11DS, MPRL40, encodes for a subunit of the mitochondrial ribosome (L. Jia, Kaur, & Stuart, 2009). iNrnms from subjects with 22qSZ have reduced levels of
mitochondrial-encoded proteins. Since MRPL40 (mitochondrial ribosomal protein L40) assists in protein translation of mitochondria encoded genes, we examined mRNA and protein levels encoded by nuclear and mitochondrial genes that generate mitochondrial proteins.

Figure 11: ATP synthase activity is unchanged between healthy control and 22qSZ

(A) Bar graph shows no significant change of ATP synthase activity in iPSC-derived neurons from 22qSZ (n=4) comparing with control (n=5). Error bars represent SEM.
(Ryan SK, Li J)

2.3.2 iNrnns from subjects with 22q11.2 deletion syndrome and SZ have reduced mtDNA-encoded protein expression

As expected, MRPL40 was reduced at the protein (Figure 12A and 12B) and at the mRNA (Figure 12I) levels (M. Lin et al., 2016). Importantly, neither protein (Figure 12A and 12B) nor mRNA (Figure 12I) levels of the nuclear
encoded voltage-dependent anion channel (VDAC) were altered in neurons from the 22qSZ group. The nuclear encoded complex II protein SDHA was also unaltered (Figure 12C and 12D), as was the mtDNA copy number (Figure 12J). Together, these results suggest that there are no gross alterations in mitochondrial mass or DNA in 22qSZ group relative to controls.

Remarkably, mitochondrial DNA-encoded protein levels, including those for MT-ND1 (complex I), cytochrome b (complex III), and COX1 (complex IV) were significantly reduced in neurons from the 22qSZ lines (Figure 12E – 12H). However, the mRNAs for these proteins were unaltered (Figure 12I). Taken together, these results suggest that the reduction of ATP in neurons from the 22qSZ-derived lines is secondary to reduced levels of mitochondrial-encoded proteins.
Figure 12: Reductions in mitochondrial-DNA encoded protein in 22qSZ iNrnns

(A&B) Protein levels, by western blot and relative to β-actin, of MRPL40, VDAC. Error bars represent SEM.
(C&D) Protein levels, by western blot and relative to β-actin, of SDHA. Error bars represent SEM.
(E&F) Protein levels, by western blot and relative to β-actin, of MT-ND1 and Cytochrome b. Error bars represent SEM.

(G&H) Protein levels, by western blot and relative to β-actin, of COX1. Error bars represent SEM.

(I) Quantitative PCR of MRPL40, VDAC, MT-ND1, Cytochrome b, and COX1 shows decreased expression of MRPL40 but no change of VDAC, MT-ND1, Cytochrome b, and COX1 in 22qSZ relative to control. Error bars represent SEM.

(J) No difference of mitochondrial DNA copy number between 22qSZ and control in iPSC-derived neurons at differentiation day (DD) 21. n=5 for control, n=4 for 22q11SZ. * indicates p<0.05. NS-not significant. Error bars represent SEM.

(Ryan SK, Li J, Deboer E)
2.3.3 Heterozygosity for a truncating mutation in MRPL40 reduces mtDNA-encoded protein expression and neuronal ATP and complex activity

(A) Schematic of CRISPR-Cas9 editing of exon 2.

Figure 13: Generation of an iPSC line haploinsufficient for mrpl40

(A) Schematic of CRISPR-Cas9 editing of exon 2.
(B) DNA sequence of the mutated line, showing four base pairs in exon 2 deleted on one allele. This 4bp deletion causes a frame shift resulting in a premature stop codon in exon 3.
(C) In iPSC cell lysates, western blotting reveals a reduction of MRPL40 but no change in VDAC in the MRPL40+/mut line. No band was detected at the predicted size of the mutated allele, possibly due to nonsense-mediated decay. (n=3).
(D) SURVEYOR detection assay is an enzyme mismatch cleavage assay, in which DNA is cut at regions of single base mismatches or small insertions or deletions. Corresponding to schematic of CRISPR-Cas9 editing of exon 2, PCR products reveal the expected 600, 480 and 120 bp bands, heterozygous mutation of MRPL40. Error bars represent SEM. (Li J)

To test whether haploinsufficiency for *MRPL40* alone is sufficient to compromise ATP levels and mitochondrial protein translation in human neurons, a loss of function mutation was introduced into one allele of *MRPL40* in an iPSC line from a healthy control (Figure 13A - 13E). iNrsns generated from this line have decreased MRPL40 protein (Figure 14A – 14D) as well as mRNA (Figure 14E). As found above with the 22qSZ iPSC-derived neurons compared to the healthy control group (Figure 12), neurons from the *MRPL40*/mut line showed no change in the nuclear encoded mitochondrial proteins VDAC or SDHA relative to its isogenic control (Figure 14A and 14B). However, MT-ND1, Cytochrome b, and COX1 were again significantly reduced at the protein level (Figure 14C and 14D) but not at the RNA level (Figure 14E). These results suggest that reduction of MRPL40 results in reduced translation of mitochondrial proteins.
Figure 14: Reduction in mitochondrial-DNA encoded proteins in a MRPL40 heterozygous line

(A&B) Western blot of MRPL40, VDAC, and SDHA from iPSC-derived neurons at differentiation day (DD) 21. MRPL40 (n=7), VDAC1 (n=5), SDHA (n=3). Error bars represent SEM.

(C&D) MT-ND1, Cytochrome b, and COX1 from iPSC-derived neurons at differentiation day (DD) 21. MT-ND1 (n=8), cytochrome b (n=6) and COX1 (n=5). Error bars represent SEM.

(E) qRT-PCR of MRPL40, VDAC, MT-ND1, Cytochrome b and COX1. In MRPL40^+/mut there is a small but significant decrease in expression of MRPL40, and no change in levels of the other transcripts. * = p<0.05, NS not significant. n=3. Error bars represent SEM.

(Ryan SK, Li J)

To determine whether the haploinsufficiency mutation for MRPL40 is also sufficient to alter mitochondrial function, ATP levels and ETC complex activities
were assessed. Similar to findings from the 22qSZ iNrns, there was a roughly 30% decrease in ATP in iNrns from the \textit{MRPL40+/mut} line (Figure 15A). Complex I and IV activities were also significantly reduced (Figure 15B and 15C), but not complexes II and III (Figure 15D and 15E). Taken together, these results suggest that \textit{MRPL40} haploinsufficiency is likely to be an important contributor to the mitochondrial phenotypes identified in iNrns from the 22qSZ group and that the reduction of ATP in iNrns from the 22qSZ-derived lines is secondary to reduced mitochondrial protein translation.

\textbf{Figure 15: Neurons derived from \textit{MRPL40+/mut} cell show decreased OXPHOS complex I and IV activity and reduced ATP level}
(A) Bar graph shows reduced ATP level in the \textit{MRPL40}^{+/mut} neurons (N=3). Error bars represent SEM.
(B-E) In \textit{MRPL40}^{+/mut} neurons there is also reduced activity in mitochondrial complex I (B, n=6) and complex IV (C, n=5), while complex II (D, n=6) and complex III (E, n=5) activity was not affected. * indicates $P<0.05$, NS-not significant. Error bars represent SEM.
(Ryan SK, Li J)

2.3.4 Neurons from subjects with 22qSZ show different cutoff frequency and similar synapse density as controls

Table 1

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</table>

Table 1: Electrophysiology properties

Electrophysiological properties between cultured neurons from the control (n=4) and 22qSZ groups (n=3). 7-10 cultured neurons were recorded in each line. $V_m$-resting membrane potential. $R_m$-input resistance, action potential (AP) /2 width, maximum firing frequency and cutoff frequency.
(Ryan SK, Li J, Goldberg EM)

The presence of reduced ATP levels in neurons from 22qSZ patients raises the question of whether these neurons have functional deficits. To test this possibility, cells were plated onto glass coverslips. Rat astrocytes, which greatly increase synaptogenesis in these cultures, were added at day 5. The media was changed to Brainphys at day 21, and then whole cell patch-clamp recordings
were performed after five weeks *in vitro* (Figure 16). Multiple parameters that could be indicative of neuronal maturational state were unchanged between the 22qSZ and controls, including resting membrane potential (Rm), input resistance, action potential half-width and max steady state firing frequency (Table 1). On the other hand, measures of parameters thought to test bioenergetics, such as cutoff frequency show significant difference in 22qSZ versus controls. In addition, synapse density, measured by confocal microscopy of immunofluorescent-labeled synapses along MAP2-labeled dendrites was not significantly different between the groups (Figure 16B and 16C). Additionally, there was no difference in the density of neurons between control and 22qSZ (Figure 16D). In sum, while the 22qSZ neurons have less tendency to respond to break through of the patch-pipette with a spike train, by multiple intrinsic electrophysiological measures as well as synapse density analysis, the 22qSZ iNrnS had well established neuronal characteristics and were not distinguishable from controls at about day 60 (Figure 16E).
Figure 16: Synaptic density in iNrs from 22q11DS with schizophrenia

(A) schema of the experimental design for the synapse density and whole cell recording at dd50.
(B) Confocal image processed by deconvolution and Imaris software showing apposition of MAP2 (Blue) with the excitatory presynaptic protein vGLUT (Green), and the excitatory postsynaptic protein PSD95 (Red) in control and 22qSZ. Scale bar: 20 µm.
(C) Bar graph shows no significant alternation of synapse density between control and 22qSZ group. (n=3). Error bars represent SEM.
(D) Bar graph shows no difference of neuron density between cultures of control and 22qSZ group in which the synapse density and whole-cell recordings were measured. \((n=3)\). Error bars represent SEM.

(E) Tracing show representative responses of neurons to 20pA current steps.

(Ryan SK, Li J, Cook K)

2.4 DISCUSSION

Schizophrenia primarily evolves from the interacting influences of multiple risk-alleles whose effects on brain development can also be influenced by both the prenatal and postnatal environment (Avramopoulos, 2018; Ursini et al., 2018). One of the strongest genetic risk factors for schizophrenia is the hemizygous microdeletion at chromosome 22q11.2 that imparts a 25% risk, or about 25 times that of the general population (Van, Boot, & Bassett, 2017). Since 9 of the roughly 45 genes deleted in 22q11DS generate proteins affect mitochondrial function (Maynard et al., 2008; Napoli et al., 2015), we examined this function in iPSC-derived neurons. We found that ATP levels were reduced in iPSC-derived neurons from patients with 22qSZ, a phenotype that appears to be caused primarily by reduced activity of complexes I and IV. Interestingly, neurons from the 22qSZ lines also had reduced levels of several mitochondrial encoded proteins, but not of nuclear encoded proteins. Since complex I and IV have the highest number of mitochondrial-encoded proteins, these findings raised the possibility that 22q11DS is associated with reduced neuronal protein synthesis, an idea supported by the presence of \(MRPL40\) in the deleted region. We thus edited a control iPSC line to be hemizygous for \(MRPL40\). Relative to its isogenic
control, and like the 22qSZ lines relative to their controls, the *MRPL40* hemizygous neurons have normal levels of mitochondria proteins encoded by nuclear genes, but decreased levels of proteins encoded by the mitochondrial DNA, decreases in ETC complex I and IV activities, and decreased ATP level. We conclude that hemizygosity at 22q11.2 is associated with decreased neuronal ATP levels, and that this phenotype is most likely related to decreased mitochondrial protein synthesis. Unfortunately, our attempts to quantify mitochondrial protein synthesis in mature iNrnns have been unsuccessful. We believe this is the first demonstration of a schizophrenia-related mutation resulting in a mitochondrial deficit in human neurons.

A key aspect of any study involving the use of human iPSCs to study disease is the cell type chosen. Here, human iPSC lines were differentiated via the induced expression of neurogenin 2, which rapidly generates a relatively homogenous population of excitatory projection neuron-like cells (iNrnns) (Yingsha Zhang et al., 2013). An advantage of this approach is that reasonably mature neurons can be generated in a matter of weeks, with high degree of consistency across lines. A disadvantage of this approach for the current study is that neurogenin 2 expression is induced by doxycycline, an inhibitor of bacterial mitochondrial protein synthesis that can also affect mammalian mitochondria (Chatzispyrou, Held, Mouchiroud, Auwerx, & Houtkooper, 2015). Of note, mitochondrial assays were run 11 days following stoppage of the doxycycline in the neuronal induction protocol, but the possibility remains that doxycycline, or even the puromycin exposure for one day at the start of the protocol, could be
unmasking a vulnerability in the 22qDS and the \textit{MRPL40} hemizygous iNrs.
Since mitochondrial gene and protein expression, as well as functional properties, are likely to differ across cell types, the relative uniformity provided by the Ngn2 protocol is another major advantage. In fact, while the lack of neuronal phenotypes such as input synaptogenesis and electrophysiological measures is disappointing, since such phenotypes could secondarily influence mitochondrial function, the high similarities in properties between iNrs from the 22qSZ and control groups strengthens our confidence that the mitochondria phenotypes themselves are not epiphenomena. That we replicated these phenotypes in the hemizygous \textit{MRPL40} line relative to its isogenic control, further bolsters this contention. In future studies, it will be important to assess mitochondrial phenotypes in longer duration culture systems, transplantations, and well as with electrophysiological conditions such as limited ATP and EGTA in the recording solution that might unmask relevant phenotypes.

It would also be interesting to study haploinsufficiency for \textit{MRPL40} affects calcium signaling, as studied in mouse hippocampal neurons (Devaraju et al., 2017). In addition, other cell types that can show schizophrenia related phenotypes should be tested, including astrocytes, microglia, and cortical interneurons. The "fast-spiking" cortical interneuron has more mitochondria in its pre-synaptic terminals than other cortical neurons (Gulyas, Buzsaki, Freund, & Hirase, 2006), and has otherwise been associated with high energy requirements, susceptibility to oxidative stress, and schizophrenia (Bitanihirwe & Woo, 2011; Steullet et al., 2017). However, to date the fast-spiking subclass of
cortical interneurons has not been definitively generated, in a highly enriched preparation, from human stem cells.

So, what bearing might these findings have to understanding schizophrenia? Of course, we cannot conclude that our findings relate to the schizophrenia-related symptoms of the 22q11.2 subjects in our study. Important results would be obtained from future studies involving the comparison of iPSCs from 22q11DS subjects with versus without SZ. It also bears mention that MRPL40 haploinsufficiency alone is unlikely to account for all mitochondrial deficits associated with 22qDS or with schizophrenia (Arion et al., 2017; Fernandez et al., 2019; Gokhale et al., 2019; Motahari, Moody, Maynard, & LaMantia, 2019), but multiple lines of evidence suggest that these findings are indeed important. First, transgenic mice hemizygous for MRPL40 show deficits in working memory, a SZ-related phenotype, as well as alterations in hippocampal short-term potentiation (Devaraju et al., 2017). Second, alterations in the expression of mitochondrial-functioning genes have been identified in a human postmortem, laser-capture microdissection study of cortical pyramidal neurons (Arion et al., 2017). Interestingly, these include downregulation of MRPL36, MRPL48, and MRPS6, raising the possibility that a mitochondrial translation deficit could be an important aspect of SZ outside of the 22q11DS context (Arion et al., 2017). In addition, MRPL18 was highlighted as a gene associated with psychosis based on whole blood RNA expression in 22q11DS subjects (Jalbrzikowski et al., 2015). Third, the capacity that we have demonstrated to study mitochondria defects in iPSC-derived neurons suggests that this system
will be amendable to increasingly refined and mechanistic studies on interactions between mitochondria genetics and neuropsychiatric risk and resilience.

3. Disruption of the Blood-Brain Barrier in 22q11.2 Deletion Syndrome

3.1 Introduction

Here we have studied the barrier and immunological properties of the CNS vasculature in the context of two models of 22q: human induced pluripotent stem cell (iPSC)-derived BBB cultures (iBBBs) from 22q patients with schizophrenia (22q+SZ) and a murine model harboring a homologous hemizygous deletion (Didriksen et al., 2017). To this end, we interrogated the barrier function, junctional protein expression and immune privilege properties of the BBB in vitro and in vivo. We further assessed the functional consequences of compromised BBB by determining the propensity of the 22q BBB to permit immune cell migration and activation in vitro and promote perivascular astrocyte activation in vivo. Our results indicate that the 22q deletion reduces BBB integrity, alters the immune privilege of the CNS vasculature, and increases the transendothelial migration of peripheral immune cells, suggesting that BBB dysfunction may contribute to the increased susceptibility to SZ in 22q patients.

3.2 METHODS

3.2.1 Human induced pluripotent stem cells (HiPSCs)

HiPSC lines were generously received from Herbert M. Lachman, MD., Einstein University, Bronx, New York (15bc4, 553c2, 1804c6, 1bc4, 60c2, 30c1, and 3113c4) and Sergiu P. Pașca, MD., Stanford University, Stanford, California (1804.5, 2788.4, 511.1). All lines were transferred over to a feeder-free system
with Stem MACS iPS-Brew XF media (Miltenyi Biotec 130-104-368). Lines were tested for mycoplasma using Lookout mycoplasma PCR detection kit (Sigma MP0035).

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Age</th>
<th>Sex</th>
<th>MLPA result</th>
</tr>
</thead>
<tbody>
<tr>
<td>553c2</td>
<td>31</td>
<td>Male</td>
<td>No deletion (HC)</td>
</tr>
<tr>
<td>1bc4</td>
<td>32</td>
<td>Female</td>
<td>No deletion (HC)</td>
</tr>
<tr>
<td>3113c4</td>
<td>21</td>
<td>Female</td>
<td>No deletion (HC)</td>
</tr>
<tr>
<td>511.1</td>
<td>27</td>
<td>Male</td>
<td>No deletion (HC)</td>
</tr>
<tr>
<td>2788.4</td>
<td>30</td>
<td>Female</td>
<td>No deletion (HC)</td>
</tr>
<tr>
<td>15bc4</td>
<td>31</td>
<td>Male</td>
<td>A-D del. (22qDS)</td>
</tr>
<tr>
<td>1804c6</td>
<td>25</td>
<td>Female</td>
<td>A-D del. (22qDS)</td>
</tr>
<tr>
<td>60c2</td>
<td>25</td>
<td>Male</td>
<td>A-D del. (22qDS)</td>
</tr>
<tr>
<td>30c1</td>
<td>41</td>
<td>Female</td>
<td>A-D del. (22qDS)</td>
</tr>
<tr>
<td>1804.5</td>
<td>23</td>
<td>Female</td>
<td>A-D del. (22qDS)</td>
</tr>
</tbody>
</table>

3.2.2 iPSC pair matches

| Healthy Control | 22qDS+SZ |
3.2.3 Differentiation of iPSCs into blood-brain barrier endothelium

iPSCs were differentiated into BBB endothelium following the protocol previously published (Hollmann et al., 2017; Lippmann et al., 2012). In brief, iPSCs were plated onto Matrigel (Corning 354230) coated 6 well TC-treated plated (Falcon 353046) at 100,000 cells/well in Stem MACs iPS-Brew XF media. The following day (Day 0) media was fully exchanged (2 mLs) to unconditioned medium (DMEM/Ham’s F12 containing 20% Knockout Serum Replacer (Invitrogen), 1× MEM nonessential amino acids (Invitrogen), 1 mM l-glutamine (Sigma), 0.1 mM β-mercaptoethanol (Sigma)). Full exchanges were performed every day through Day 5. On day 6, media was fully exchanged (4 mLs) to endothelial cell (EC) medium with retinoic acid (human Endothelial Serum-Free Medium (Invitrogen 11111), 1% platelet-poor plasma-derived bovine serum (Biomedical Technologies 50-443-029) 20 ng/mL bFGF (Peprotech 100-18B), and 10µM retinoic acid (Sigma R2625). An addition 2 mLs of EC medium with retinoic acid
was added on day 7. On Day 8, induced BBB (iBBB) endothelial cells were frozen down.

3.2.4 Cryopreservation of iBBB
On Day 8 of differentiation, iBBB were washed twice with PBS (Phosphate-buffered saline) (1X), then lifted with StemPro accutase (ThermoFisher Scientific A11105-01) and spun down at 1,000 rpm for 5 min. The supernatant was aspirated, and the cells were re-suspended in 60% EC medium with retinoic acid, 30% FBS (fetal bovine serum) (Hyclone SH30071.03HI), and 10% DMSO at 2 x 10^6 cells/mL. Cells were stored in liquid nitrogen.

3.2.5 iBBB culturing
Plates were coated with a collagen/fibronectin mixture composed of 50% sterile H2O, 40% collagen from human placenta (Sigma C5533), and 10% fibronectin from bovine plasma (Sigma F1141) by volume as previously described (Hollmann et al., 2017; Lippmann et al., 2012). Cells were counted and plated according to Table 1 in EC medium (human endothelial Serum Free Media (Invitrogen) with 1% platelet-poor plasma-derived bovine serum (Alfa Aesar J64483)) with 20 ng/mL bFGF (Peprotech), 10 μM retinoic acid (Sigma), and 1:1000 Y27632 (ROCK inhibitor) (R&D 1254) overnight. Media was changed 24 hrs later to EC medium, and subsequently changed every 48 hours after, allowing cells to grow at 37°C in 5% CO2 until confluency was reached, for a maximum of 5 days. All experiments were conducted in paired format, in which 22q+SZ and age/sex matched HC were thawed, plated and analyzed in parallel.
All iBBB data is presented as color/shape-matched pairs as follows: 15bc4/553c2, red circle; 30c1/3113c4, gray square; 60c2/511.1, blue diamond; 1804.5/2788.4, green triangle; 1804c6/1bc4, purple hexagon.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Cell Density</th>
<th>Media Volume</th>
<th>Plating Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEER</td>
<td>1.75 x 10⁵</td>
<td>400 μL</td>
<td>8W10E+ electrode arrays (Applied Biophysics, Troy, NY, USA)</td>
</tr>
<tr>
<td>Immunofluorescence</td>
<td>8 x 10⁴</td>
<td>300 μL</td>
<td>8 well chamber slide (ibidi)</td>
</tr>
<tr>
<td>Flow cytometry</td>
<td>1.8 x 10⁵</td>
<td>2 mL</td>
<td>Nunclon™ Delta Multidishes, 6 well (ThermoFisher Scientific)</td>
</tr>
<tr>
<td>Western blot</td>
<td>1.8 x 10⁵</td>
<td>2 mL</td>
<td>Nunclon™ Delta Multidishes, 6 well (ThermoFisher Scientific)</td>
</tr>
<tr>
<td>Monocyte migration</td>
<td>8 x 10⁴</td>
<td>(Bottom) 1.5 mL, (Top) 600 μL</td>
<td>Falcon cell culture inserts (ThermoFisher Scientific)</td>
</tr>
</tbody>
</table>

3.2.6 iBBB TEER
The electrical properties of confluent monolayers of iBBB endothelial cells were measured as previously described (Alvarez, Dodelet-Devillers, et al., 2011). In brief, the Electric Cell-substrate Impedance Sensing (ECIS) methodology was
employed making using of the ECIS Z instrument and 8W10E+ electrode arrays (Applied Biophysics, Troy, NY, USA). The TEER properties of iBBB cell monolayers were recorded at 2000 Hz over a period of 72 hrs. BBB-ECs were inoculated at $1.75 \times 10^5$ cells/well in 400 μL of media and in triplicates.

### 3.2.7 iBBB immunofluorescence

Cells were washed twice with TBS (1X) (Tris-buffered saline) and fixed with 70% Ethanol for 5 min. Unspecific antibody binding was blocked for 30 min with 10% normal donkey serum (Sigma) followed by overnight 4°C incubation with rabbit polyclonal antibodies against claudin-5 (1:100; Life Technologies) and ZO-1 (1:200; Life Technologies) diluted in 3% normal donkey serum. After multiple washes in TBS (1X) with 0.025% Tween 20 (Amresco), secondary antibody Alexa Fluor® 594 AffiniPure Fab Fragment Donkey Anti-Rabbit IgG (1:300; Jackson Immunoresearch) was added and incubated for 60 minutes at room temperature. iBBB monolayers were then washed twice in TBS (1X) with 0.025% Tween 20 and mounted with Gelvatol containing Hoescht (1:1000; Invitrogen) for nuclear staining. Cells were imaged using a Leica SP5 confocal microscope (Leica Microsystems). Images were obtained using an Olympus IX83 set up for brightfield and fluorescence, and equipped with a motorized X, Y, Z stage and a spinning disk confocal head (X-Light V2, Crestoptics s.r.l., Rome, Italy) using a Hamamatsu R2 cooled CMOS camera (Hamamatsu City, Japan) operated by the MetaMorph software (Molecular Devices, LLC, Sunnyvale, CA).
3.2.8 iBBB flow cytometry
Following 5 days in vitro, iBBB cells were detached with StemPro accutase (ThermoFisher Scientific A11105-01) and nonspecific binding was blocked with Mouse IgG Isotype Control (Invitrogen) for 25 minutes at 4°C. Cells were stained with monoclonal mouse anti-human ICAM-1 (1:100; Biolegend HA58) for 30 minutes 4°C, and data was collected on LSRFortessa (BD Biosciences). Mean fluorescent intensity (MFI) of the ICAM-1+ population was measured with FlowJo version 10 software (BD biosciences). ICAM-1 expression in each pair was analyzed in 2-4 separate experiments, and significance was determined using the Wilcoxon matched-pairs signed rank test in GraphPad Prism 7.04.

3.2.9 iBBB western blot
Following 5 days in vitro, cells were detached with StemPro accutase (ThermoFisher Scientific A11105-01), incubated in RIPA Lysis buffer (Amresco) with 1:100 protease inhibitor (Sigma) on ice for 30 minutes and subsequently centrifuged. Protein content quantified using the Pierce™ BCA Protein Assay kit according to manufacturer protocols (Thermo Fisher Scientific). 20 μg of protein lysate was run on 4-20% Mini-PROTEAN TGX Precast Gel (BioRad), transferred to nitrocellulose membrane (BioRad) and blocked with Odyssey Blocking Buffer (Licor) for 1 hour at room temperature. Primary polyclonal rabbit antibodies against claudin-5 (1:500; Life Technologies) and ZO-1 (1:250; Life Technologies) were diluted in Odyssey Blocking Buffer and incubated overnight at 4°C. The membrane was washed with TBS (1X) with 1% Tween 20 (Amresco), secondary antibodies IRDye 680RD donkey anti-mouse and IRDye 800CW donkey anti-
rabbit (Licor) were diluted 1:5000 in Odyssey Blocking Buffer and incubated 1 hour at room temperature. Standard monoclonal mouse anti-β actin (1:10000; Sigma AC-74) was incubated 1 hour at room temperature. The membrane was developed on Odyssey Infrared Imaging System 9120 (Licor), and average pixel intensity of each band was measured by ImageJ.

3.2.10 Immune cell transmigration across iBBB monolayers
To investigate monocyte migration across the 22q BBB, we utilized a transwell model in which iBBB cells were grown on the upper chamber of 6.4 mm filter Falcon cell culture inserts (ThermoFisher Scientific) for 5 days. Monocytes were isolated from the blood of 8 healthy volunteers as previously described (Kebir et al., 2009). In brief, peripheral blood mononuclear cells were isolated using density gradient centrifugation on Ficoll-Paque™ (GE Healthcare). CD14⁺ cells were obtained using the MACS isolation columns according to the manufacturer's protocol (Miltenyi). CD14⁺ cells (1×10⁶ per well) were allowed to migrate for 18 hours, and migrated monocytes were studied using flow cytometry immunostaining. Nonspecific binding was blocked with Mouse IgG Isotype Control (Invitrogen) for 25 minutes at 4°C. Cells were stained with mouse anti-human CD14 (Biolegend HCD14 in PerCP), mouse anti-human HLA-DR (Biolegend L243 in Alexa Fluor® 700), and mouse anti-human CD141 (Biolegend M80 in PE-Cy7) diluted 1:100 for 30 minutes at 4°C, and data was collected on LSR Fortessa (BD Biosciences). Analysis was performed with FlowJo version 10 software (BD biosciences). Each HC/22q+SZ iBBB pair was run 1-2 times with
monocytes isolated from a different healthy volunteer. CD141 expression was evaluated in the CD14+ MHCII+ positive population, and significance was determined using the Wilcoxon matched-pairs signed rank test in GraphPad Prism 7.04.

3.2.11 Mice
All experiments were conducted in accordance with IACUC at the University of Pennsylvania. 4-6-month-old male and female 22q mice and WT littermates (Taconic) were anesthetized by either isofluorane or CO₂ and transcardially perfused with ice cold PBS (1X). Brains either frozen in Optimal Cutting Temperature (OCT) Compound (Fisher HealthCare) for sectioning, kept fresh in RPMI (1X)+GlutaMAX (Gibco) for flow cytometry or frozen at -80°C for western blotting.

Mouse anterior cortex was thawed and sonicated in RIPA Lysis buffer (Amresco) on ice using an Ultrasonic Homogenizer (Biologics Inc.). Western blot analyses proceeded as described above, and significance was determined by unpaired student’s t test in GraphPad Prism 7.04.

3.2.12 Mouse CNS endothelial cell flow cytometry
Microvascular endothelial cells were isolated from the anterior portion of the CNS for flow cytometry analysis as previously described (Crouch & Doetsch, 2018). In brief, tissue was minced in HBSS (1X) (Hank’s Balanced salt solution, ThermoFisher Scientific) containing 1% bovine serum albumin (Fisher
BioReagents), 0.1% glucose (Sigma) and 0.5 mg/mL DNase I (Roche) and was subsequently digested with 3 mg/mL Collagenase/Dispase (Roche) in PBS (1X) with 2% FBS (Serum Solutions International) for 30 minutes at 37°C with agitation. Following centrifugation, cells were resuspended in Trituration solution (PBS (1X) with 2% FBS and 0.5 mg/mL DNase I) and endothelial cells were obtained using density gradient centrifugation with 22% Percoll (GE Healthcare) in PBS (1X). Nonspecific binding was blocked with rat anti-mouse CD16/32 (1:100; Biolegend 93) for 20 minutes at 4°C, and cells were stained with rat anti-mouse ICAM-1 (1:100; Biolegend YN1/1.7.4 in PerCP), rat anti-mouse CD45 (1:100; BD Horizon™ 30-F11 in Brilliant Violet 510), rat anti-mouse CD102 (1:100; Biolegend 3c4 (MIC2/4) in Alexa Fluor® 488) and rat anti-mouse CD31 (1:100; Biolegend 390 in APC/Fire™ 750) for 30 minutes at 4°C. Cells were fixed in 4% PFA (paraformaldehyde) for 10 minutes at room temperature, and data was collected on the LSRFortessa (BD Biosciences). MFI of the ICAM-1-positive endothelial (CD45^- CD102^ CD31^) population was measured with ImageJ version 10 (BD Biosciences). Data was normalized to the average of the WT(s) in each experiment, across 4 different experiments (n=6 males), and significance was determined using unpaired student's t test in GraphPad Prism 7.04. Females were excluded due to low numbers (in 1 experiment, n=2 WT, 1 22q).

3.2.13 Immunohistofluorescence
8 μm sagittal brain sections were fixed with ice cold acetone and 70% ethanol, permeabilized with TBS (1X) with 0.025% Tween 20 (Amresco), and nonspecific
binding was blocked with 10% normal donkey serum (Sigma) for 4 hours at room
temperature. Primary antibodies polyclonal rabbit anti- rat/mouse fibrinogen
(1:300; Innovative research), polyclonal rabbit anti-mouse/human claudin-5
(1:300; Life Technologies), polyclonal rabbit anti-mouse/human ZO-1 (1:200; Life
Technologies), monoclonal rat anti-mouse ICAM-1 (1:100; Biolegend YN1/1.7.4),
monoclonal mouse anti-mouse GFAP (1:2000; Sigma G-A-5), and monoclonal
rat anti-mouse IL-6 (1:50; Invitrogen, MP5-20F3) were diluted in 3% normal
donkey serum and incubated overnight at 4°C. Sections were washed with TBS
(1X), incubated with secondary antibodies diluted 1:300 (Alexa Fluor® 488
AffiniPure F(ab')₂ Fragment Donkey Anti-Rabbit IgG, Alexa Fluor® 488 AffiniPure
Donkey Anti-Rat IgG, Rhodamine Red™-X (RRX) AffiniPure F(ab')₂ Fragment
Donkey Anti-Mouse IgG; Jackson Immunoresearch) and Isolectin GS-IB4 From
Griffonia simplicifolia, Alexa Fluor® 647 Conjugate (1:300, Life Technologies) in
3% normal donkey serum (Sigma) for 2 hours at room temperature. Nuclei were
permeabilized with TBS (1X) with 1:100 Triton X-100 (Amresco) for 10 minutes
and mounted with Gelvatol containing Hoescht nuclear dye (1:1000; BD
biosciences). Sections were imaged on Leica widefield microscope (Leica
Microsystems) at 10x (GFAP tilescans), 20x (IL-6/GFAP, fibrinogen/IgG) and 40x
(claudin-5, ZO-1, ICAM-1). All immunofluorescent analyses were performed
blinded and using ImageJ as previously described (Alvarez et al., 2015).
Extravascular fibrinogen and IgG in the anterior cortex was quantified as the
average pixel intensity of the leakage area; 12 vessels were analyzed from four
20x z stack maximum projections per animal. Transected vessels stained for claudin-5 and ZO-1 in the anterior cortex were imaged in four 40x z-stack maximum projections per animal. Average pixel intensity was measured at 3 points along each TJ strand and were averaged to a single measurement per TJ; 5 TJ strands from 5 different vessels were analyzed per animal. Vascular ICAM-1 expression was imaged in the anterior cortex in 3 40x z-stack maximum projections per animal. Maximum pixel intensity was obtained at 3 points along each of the four brightest ICAM-1 positive vessels in each animal, and these were averaged to find the average pixel intensity of ICAM-1 per blood vessel. GFAP and IL-6 expression was imaged at 20x in the hippocampus; IL-6 expression was quantified as the maximum pixel intensity per astrocyte process, with a total of 10 processes analyzed per animal. N=10 WT and 10 22q (14 females, 6 males from 3 experiments) except assessment of barrier function (fibrinogen/IgG, Figure 1), in which mice noted with poor CNS perfusion at time of brain harvest were excluded. Significance was determined by unpaired student’s t test using GraphPad Prism 7 for all.

3.2.14 Human postmortem samples
Using a clinical database of patients with 22q deletion syndrome, three subjects with full postmortem neuropathological examinations were identified for inclusion in this study (ages: 1, 2 and 14 months; all patients were female). A pathology database was queried to identify post-mortem samples from age-matched controls. Three controls with congenital heart disease (ages: 1, 1, and 16
months; M/F: 2/1) and two without congenital heart disease (ages: 1 and 12 months; all female) were included. All materials were obtained from pre-existing formalin-fixed, paraffin-embedded tissue of the frontal lobe and were histologically confirmed to contain both cortex and white matter. All materials from human subjects were de-identified in accordance with the Children's Hospital of Philadelphia Institutional Review Board requirements.

3.2.15 Immunohistofluorescence of human brain samples
Immunostaining of paraffin-embedded brains was performed as previously published. In brief, sections from postmortem controls (n=3) and 22q patients (n=3) were sectioned and mounted on positively charged slides (ThermoFisher Scientific). Sections were deparaffinized by washing in xylene followed by washing in 100%, 95% and 70% ethanol following by H₂O. Epitope retrieval was performed in a microwave using citrate buffer pH 6.0 (Dako) for 30 minutes. Tissue sections were permeabilized with wash buffer (Dako) for 5 minutes and blocked at room temperature for 90 minutes in 10% normal donkey serum (Sigma). Antibodies were diluted in 3% normal donkey serum (monoclonal mouse anti-mouse GFAP 1:2000, Sigma G-A-5; polyclonal rabbit anti-human IL-6 1:50, Proteintech), and incubated overnight at 4°C. Secondary antibody Alexa Fluor® 488 AffiniPure F(ab’)₂ Fragment Donkey Anti-Rabbit IgG was diluted 1:300 in 3% normal donkey serum, incubated 30 minutes at 37°C, and mounted with Gelvatol containing Hoescht nuclear dye (1:1000; BD Biosciences). Confocal microscopy was performed in an Olympus IX83 as indicated above.
3.3 RESULTS

3.3.1 Barrier function is impaired in the 22q BBB

As the barrier function of CNS vascular endothelial cells underlies the ability of the BBB to restrict peripheral influences on CNS function (Abbott et al., 2010), we first assessed barrier properties of the 22q BBB. To evaluate the effect of the 22q deletion on barrier function, we obtained iPSCs from 5 22qSZ patients and 5 paired age- and sex- matched healthy controls (HC). iBBB endothelial cells were derived from these iPSCs using a differentiation protocol as previously described (Figure 17A) (Hollmann et al., 2017; Lippmann et al., 2012). To interrogate the barrier function of the iBBB monolayers, we assessed their transendothelial electrical resistance (TEER) by repeatedly sampled resistance of the monolayers over the course of 4-6 days. We observed a significant decrease in TEER of the 22qSZ iBBB monolayers at confluency compared to paired HC monolayers (Figure 17B), indicating that barrier function of the BBB is compromised in 22q (Figure 17C).

To evaluate the 22q BBB in vivo, we utilized a mouse strain harboring a hemizygous deletion of the 22q homologous region on chromosome 16 (Didriksen et al., 2017). These mice mimic much of the biology of 22q in humans, including facial deformities and SZ-associated behavioral changes (Didriksen et al., 2017). We assessed BBB integrity by quantifying extravasation of blood proteins into the CNS parenchyma of 22q mice and their wild type (WT)
littermates. Vasculature was cleared by transcardial perfusion, and immunofluorescent analyses demonstrated a significant increase in extravascular leakage of two serum proteins, immunoglobulin G (IgG) and fibrinogen (Figure 17D - 17E) in 22q. Together, our results support the hypothesis that the BBB is intrinsically compromised in 22q.
Figure 17: 22q BBB demonstrates compromised barrier properties
(A) iPSCs were differentiated into BBB endothelial cells as previously described; differentiated cells were grown in conditioned endothelial cell media for 24 hours followed by 5 days culture in unconditioned endothelial cell media prior to experimental analysis.
(B) Representative TEER curve for one pair of HC and 22q+SZ iBBB cells, run in triplicate
(C) Quantitative analysis of iBBB TEER data as a function of fold change across each pair (n=5 pairs, 2-4 replicates per pair, **, p<0.01)
(D) Representative immunofluorescent image of IgG (green) in the CNS of WT and 22q mice. White arrows indicate extravascular leakage of IgG. Scale bar represents 50µm.
(E) Quantitative analysis of perivascular extravasation of serum proteins IgG and fibrinogen in the PFC of WT and 22q mice (n= 8 WT, 10 22q, 12 vessels per animal; ***, p<0.001, **** p<0.0001 by unpaired t test)
(Crockett AM, Ryan SK)

3.3.2 Claudin-5 expression is compromised in the 22q BBB

As our TEER results indicate impaired junctional integrity underlying compromised barrier function, we next analyzed tight junction protein expression in the 22q BBB. Because claudin-5 is the most densely expressed tight junction molecule in the BBB (Morita, Sasaki, Furuse, & Tsukita, 1999; Ohtsuki et al., 2007; Ohtsuki, Yamaguchi, Katsukura, Asashima, & Terasaki, 2008) and the gene for claudin-5 is included in the 22q deletion (Arinami, 2006; Greene et al., 2018), we assessed claudin-5 expression in our iBBB endothelial cell cultures. There was no change in claudin-5 protein level between 22qSZ and HC cultures according to western blot (Figure 18A and 18B), suggesting that compromised barrier integrity in 22q+SZ iBBB monolayers is not solely due to insufficient claudin-5 gene dosage. As the organization of tight junctions is critical for proper barrier function, we assessed claudin-5 localization in the paracellular cleft
between endothelial cells and observed highly disorganized expression in 22q+SZ iBBI cells (Figure 18C).
Figure 18: Claudin-5 expression is disrupted in the 22q BBB
In contrast to *in vitro* claudin-5 expression, we observed reduced levels of claudin-5 in the brains of 22q mice compared to WT by both western blot (Figure 18D and 18E) and immunofluorescence (Figure 18F and 18G). This deficit was specific to claudin-5, as we did not observe changes in ZO-1 expression by either western blot or immunofluorescence (Figure 19A – 19D). Thus, claudin-5 expression is impaired in the 22q BBB.

### 3.3.3 Immune privilege properties are affected in the 22q BBB

Given that the BBB serves as an immunological boundary between the periphery and the CNS (Abbott et al., 2010; Engelhardt & Coisne, 2011; Muldoon et al., 2013), and because of the renewed focus on the immune system in SZ (Miyaoka et al., 2017; Muller, 2018; Pollak et al., 2018; Severance et al., 2016; Wei & Hemmings, 2005), we assessed the immunoquiescent properties of the 22q BBB. We evaluated expression of intercellular cell adhesion molecule 1...
(ICAM-1; CD54), a cell adhesion molecule upregulated by the BBB upon inflammation (Alvarez et al., 2015; Dietrich, 2002; Muldoon et al., 2013) and in the post mortem SZ BBB (Cai et al., 2018). We found elevated ICAM-1 expression in 22q+SZ iBBB endothelial cells compared to HC pairs (Figure 20A and 20B), suggesting immune activation of the 22q BBB endothelium.

Figure 19: ZO-1 expression is not disrupted in 22q mouse brains

(A&B) Western blot (A) and quantitative analysis (B) of ZO-1 expression in mouse brains. (n=11 WT brains, n=10 22q brains). Unpaired t test, n.s. = not significant. Error bars represent SEM.

(C) Representative immunofluorescent image of ZO-1 (green) distribution in WT and 22q mouse PFC. Scale bar represents 20µm.
(D) Quantitative analysis of ZO-1 expression in the PFC of WT and 22q mice (n=10, 5 TJs per mouse, unpaired t test).
(Crockett AM)

To assess the immunological properties of the 22q BBB in vivo, we isolated vascular endothelial cells from the brain of WT and 22q mice and determined their cell adhesion molecule expression by flow cytometry. As in our iBBB cultures, we again observed a significant increase in ICAM-1 expression on the CNS vasculature in 22q (Figure 20C and 20D). In situ analysis of 22q and WT PFC sections further confirmed elevated ICAM-1 expression on the 22q BBB, indicating alterations in the inflammatory status of the 22q BBB (Figure 20E and 20F). Thus, immune privilege properties of the CNS vasculature are impaired in 22q.
Figure 20: ICAM-1 expression is elevated in the 22q BBB

(A) Representative histogram plot of ICAM-1 expression on HC and 22q+SZ iBBB endothelial cells.
(B) Flow cytometry analysis of ICAM-1 expression on HC and 22q+SZ iBBB endothelial cells (n=5 pairs, each pair analyzed in 2-4 different experiments, data represented by colors corresponding to each pair of donors; *, p < 0.05 by Wilcoxon matched-pairs signed rank test).
(C) Representative histogram plot of ICAM-1 expression on CNS endothelial cells from a WT and a 22q mouse.
(D) Flow cytometry analysis of ICAM-1 expression on CNS endothelial cells from the PFC of WT and 22q mice (n=6; *, p < 0.05 by unpaired t test).
(E) Representative immunofluorescent image at 20x (L) and 60x (R) of ICAM-1 (green) expression in vasculature (red) of the PFC in WT and 22q mice. Scale bar represents 20µm.
(F) Quantitative analysis of vascular ICAM-1 expression in the PFC of WT and 22q mice (n=10, 4 vessels per mouse; * p < 0.05 by unpaired t test).
(Crockett AM, Ryan SK)

3.3.4 22q BBB promotes monocyte migration and activation

To assess the functional consequences of compromised barrier function and immune properties of the 22q BBB, we migrated human immune cells across iBBB endothelial cell monolayers (Figure 21A). We found that iBBB monolayers derived from 22qSZ patients were less able to restrict migration of human monocytes (Figure 21B). To determine the effect of transendothelial migration, we assessed monocyte phenotype post-migration (Figure 21C). We found that following migration across 22qSZ iBBB monolayer, monocytes significantly decreased thrombomodulin (TM, CD141) expression (Figure 21D and 21E). As TM is known to be an anti-inflammatory molecule expressed on both immune cells and the luminal endothelium to inhibit immune cell adherence and extravasation (Griffin et al., 2006; Loghmani & Conway, 2018; H. Takahashi et al., 1992; van de Ven, Lindenberg, Oosterhoff, & de Grujil, 2013; Xu et al., 2015), our findings suggest that transmigration across the 22qSZ BBB has a pro-migratory effect on immune cells.
Figure 21: 22q BBB promotes immune cell transmigration and activation

(A) Workflow for isolation and migration of CD14+ monocytes.
(B) Number of monocytes migrating across the iBBB (n=5 pairs, 2 replicates per pair, each within-pair replicate performed with monocytes isolated from a different healthy volunteer for all, data represented by colors corresponding to each pair of iBBB donors; p < 0.05 by Wilcoxon matched-pairs signed rank test).
(C) Gating strategy for analysis of transmigrated CD14+ monocytes.
(D) Representative flow plots of TM expression on transmigrated monocytes.
(E) Flow cytometry analysis of TM expression on transmigrated monocytes (p < 0.01 by Wilcoxon matched pairs signed rank test).
(Crockett AM, Ryan SK)

3.3.5 Perivascular astrocytes are activated in 22q

We next aimed to assess the functional consequences of compromised barrier function in 22q mice. As astrocytic endfeet ensheath the CNS vasculature (Abbott et al., 2006; Cheslow & Alvarez, 2016), and because we have found elevated perivascular fibrinogen, a molecule that is highly pro-inflammatory in the CNS (Davalos et al., 2012; Muradashvili, Tyagi, & Lominadze, 2017; Schachtrup et al., 2010), we evaluated astrocyte activation by glial fibrillary acidic protein (GFAP) expression. We observed upregulation of GFAP in the brains of 22q mice compared to WT, indicating widespread astrocyte activation. Interestingly, this astrocyte activation appeared to be mainly along the CNS vasculature of the meninges (Figure 22A). In order to characterize the activation of astrocytes in 22q, we focused our analyses on the pro-inflammatory cytokine IL-6, as this molecule has been repeatedly shown to be elevated in the blood of both idiopathic SZ patients and 22qSZ patients (G. M. Khandaker, Pearson, Zammit, Lewis, & Jones, 2014; Mekori-Domachevsky et al., 2017; Potvin et al., 2008; Subbanna et al., 2018). We found that IL-6 expression was significantly
upregulated in astrocytes bordering large CNS vessels of the meninges in 22q mice (Figure 22B), suggesting that this activation may not be solely due to intrinsic astrocyte defects, but rather a consequence of compromised barrier function in the 22q CNS.

To determine the status of neurovascular astrocytes in human 22q patients, we obtained postmortem brain sections from 3 22q patients and 3 age-matched HCs. Immunofluorescent staining for GFAP and IL-6 indicated elevated astrocyte activation and IL-6 expression in 22q patients compared to HCs (Figure 22C and 22D). Together this data indicates that neuroimmune vascular activation in 22q involves the astroglial compartment.
Figure 22: Perivascular astrocytes are activated in 22q

(A) Representative sagittal sections of WT and 22q brains stained for GFAP (red, bottom) and hippocampal meninges GFAP (red) and IL-6 (green) expression (40x, top).

(B) Quantitative analysis of IL-6 expression along hippocampal meninges (n=10, 10 astrocytes per mouse, **** p < 0.0001 by unpaired t test).

(C) Representative immunofluorescent images of human 22q and HC postmortem brain sections stained for GFAP (red) and IL-6 (green) at 20x (right) and 40x (middle, left). White arrows indicate parenchymal IL-6+
3.4 DISCUSSION

For the first time, our results indicate BBB dysfunction in the context of 22q, the strongest monogenic risk allele for SZ (Gur et al., 2017). These results are supported by clinical data suggesting that barrier function and immune privilege properties of the BBB are affected in idiopathic SZ patients. Multiple studies have reported that SZ is associated with elevated serum proteins such as immunoglobulins and albumin in the cerebrospinal fluid (CSF), consistent with compromised barrier function (Endres et al., 2015; Severance et al., 2015). Similarly, we have found increased extravasation of serum proteins into the CNS parenchyma in 22q mice compared to WT, and this finding of compromised barrier function is further supported by impaired TEER in our 22q+SZ iBBB monolayers.

Polymorphisms in the claudin-5 gene have been associated with SZ (Greene et al., 2018; Omidinia, Mashayekhi Mazar, Shahamati, Kianmehr, & Shahbaz Mohammadi, 2014; Z. Y. Sun et al., 2004; Wu et al., 2010), including recent work by our lab demonstrating an association between the claudin-5 rs10314 variant and SZ diagnosis in a large cohort female 22q patients. Furthermore, claudin-5 mRNA and protein expression has been reported to be decreased in the post mortem SZ brain compared to controls (Nishiura et al.,
To our surprise, our 22q+SZ and HC iBBBs expressed indistinguishable levels of claudin-5 protein, while 22q mice expressed significantly less claudin-5 than WT. Rather, altered claudin-5 organization within the paracellular cleft of endothelial cells appears to compromise barrier function in 22q+SZ iBBBs. This contrast between *in vivo* and *in vitro* data suggests that the effects of the 22q deletion on barrier function are not solely due to claudin-5 gene dosage, but more likely due to an interaction of genes in the deleted region or an exaggerated stress response *in vitro*. Furthermore, we have found that ICAM-1 is upregulated in 22q, implicating differences in inflammatory/stress responses in 22q (Alvarez, Cayrol, et al., 2011; Dietrich, 2002). Alternatively, other factors *in vivo*, including circulating pro-inflammatory molecules and/or the close association with astrocyte endfeet (Abbott, 2000; Abbott et al., 2006; Cheslow & Alvarez, 2016), may alter the effects of the deletion on the BBB *in vivo* compared to *in vitro*.

In addition to compromised barrier function of the BBB, it has also been reported that immunoquiescent properties of the CNS vasculature may be disrupted in SZ patients (T. T. Nguyen et al., 2018). Postmortem studies have indicated elevated expression of the pro-inflammatory cell adhesion molecule ICAM-1 in the CNS endothelium of SZ patients (Cai et al., 2018), which may contribute to the increased presence of peripheral immune cells within the CNS parenchyma in post mortem SZ brains (Golam M. Khandaker et al., 2015). We have also found an increase in ICAM-1 expression in both our 22q+SZ iBBB cells and in vascular endothelial cells isolated from the CNS of 22q mice. This may
contribute to the increase in migration of monocytes across the 22q+SZ iBBB monolayer. We postulate that the increase in ICAM-1 expression in 22q mice may represent an increased immune sensitivity to facilitate leukocyte migration into the CNS upon environmental stressor, such as infection or injury.

Using our 22q mice, we were able to probe beyond the BBB to interrogate the status of the neurovascular unit. As we had found compromised barrier function in vivo and because fibrinogen is known to be a highly pro-inflammatory molecule in the CNS, we hypothesized that this may contribute to astrocyte activation. Our observations build upon previous findings of astrogliosis in the post mortem 22q brain (Kiehl, Chow, Mikulis, George, & Bassett, 2009) and we report our novel finding that astrocyte activation is associated with significantly increased astrocytic IL-6 expression in 22q. Notably, we observed that IL-6 expression was elevated primarily in astrocytes lining the meningeal CNS vasculature in 22q mice. Although we cannot attribute our observations of astrogliosis in 22q mice specifically to compromised BBB, the pattern of astrocyte activation predominantly along large CNS vessels suggests that impaired barrier function may contribute to astrocyte-mediated neuroinflammation in 22q (Muradashvili et al., 2017; Schachtrup et al., 2010; Verkhratsky & Nedergaard, 2018). Subsequent studies on the response to environmental challenges will address the intrinsic immune susceptibility of the CNS in 22q.

It is important to note that in both our human 22q+SZ iBBB cultures and our human 22q+SZ brain sections, we cannot determine the role of SZ diagnosis
in our results. It is unclear whether all 22q patients present with compromised barrier function, impaired claudin-5 structure, and elevated ICAM-1 expression, which together may contribute to the increased risk for neuropsychiatric conditions in this population, or whether these results are only evident in 22q patients that develop SZ. As our murine data indicate BBB dysfunction in naïve 22q mice, we hypothesize that our results are conferred by the deletion alone, regardless of SZ diagnosis, and therefore may contribute to the increased susceptibility to SZ and other neuropsychiatric disorders in 22q (Fiorentino et al., 2016; Gandal et al., 2018; Kealy et al., 2018), but future studies including a 22q without SZ group will be important to address these questions.

This is the first study to assess the status of the BBB in the context of 22q, a population known to have a 25% increased risk of developing SZ (Arinami, 2006; Karayiorgou & Gogos, 2004; Van et al., 2017). Our results support the hypothesis that neuroinflammation and compromised BBB may play a role in the pathogenesis of neuropsychiatric disorders. Fundamental to this hypothesis is the clinical data indicating peripheral inflammation in a subpopulation of SZ patients, including elevated peripheral cytokines (most notably IL-6) and immune cell activation (especially of Th17 cells) (Debnath & Berk, 2014; Potvin et al., 2008; Subbanna et al., 2018). Interestingly, 22q patients present with a similar inflammatory profile, as they also have elevated peripheral IL-6 and increased percentages of circulating Th17 cells (Mekori-Domachevsky et al., 2017; O'Rourke & Murphy, 2019; Vergaelen et al., 2018). Thus, our results suggest that
22q patients may represent a uniquely appropriate population to assess the role of peripheral inflammation and BBB dysfunction in neuropsychiatric disorders like SZ.

4. Neuroinflammation and EIF2 signaling persist despite antiretroviral treatment in an HiPSC tri-culture model of HIV infection

4.1 Introduction

We have developed a Human-induced pluripotent stem cell (HiPSC) based model whereby we separately differentiate HiPSCs into forebrain-like excitatory neurons, astrocytes, and microglia then combine them to create a tri-culture, with or without HIV-infection of the microglia, and with or without ART. Our protocol rapidly produces microglia-like cells (iMg) that express multiple classical markers in mono-culture, productively infect with HIV, and respond to ART. In addition, we have developed a differentiation protocol for astrocyte-like cells (iAst) that express hallmark proteins. Utilizing this system, we investigated the effects of HIV infection (Inf), infection with the antiretroviral Efavirenz (EFZ) (Inf+EFZ), and EFZ treatment alone (Uninf+EFZ), compared to uninfected tri-cultures (Uninf) and to each other. Interestingly, acute HIV Inf reduced synaptophagy by both infected iMg and uninfected iMg in the cultures. Inf also caused gene expression changes consistent with highly activated inflammatory cytokine signaling in iMg, and activation of EIF2 signaling in iMg, iAst, and iNrn. While Inf+EFZ reduced many inflammatory markers, EIF2 signaling activation persisted in the iNrnms and RhoGDI and CD40 signaling persisted in the iMg, of the Inf+EFZ condition. In addition, EFZ treatment alone invoked its own discrete inflammatory response, reaffirming the toxic effects of EFZ (Ciccarelli et al., 2011) and revealing novel pathways that may contribute to the toxicity. Our system, which recapitulates key
findings in patient studies, provides a platform to mechanistically understand responses to HIV seen in human studies and further reveal the complex roles of the individual cell types during infection ± ART, how ART alone can elicit an inflammatory response, and the prominent role microglia play in the early inflammation response to HIV infection in the brain.

4.2 Methods

4.2.1 Human induced pluripotent stem cell lines
HiPSC lines for the iNrn and iAst were generously received from Herbert M. Lachman, MD., Einstein University, Bronx, New York. All lines were trained over to a feeder-free system with Stem MACS iPS-Brew XF media (Miltenyi Biotec 130-104-368). Lines were tested for mycoplasma using Lookout mycoplasma PCR detection kit (Sigma MP0035). iPSC lines for the iMg were cultured by the Human Pluripotent Stem Cell Core (CHOP).

4.2.2 iNeuron differentiation of iPSCs
iPSCs were transfected with two plasmids VSVG.HIV.SIN.cPPT.CMV.mNgn2.WPRE and VSVG.HIV.SIN.cPPT.CMV.rTTA.WPRE, produced by Marius Wernig (Stanford University) and packaged by the University of Pennsylvania Viral Vector Core. Cells were exposed to 1μg/mL polybrene (Sigma Aldrich TR-1003). Media is fully exchanged six hours after exposure. iPSCs are differentiated according to a previously published method (Zhang et al., 2013). In brief, after transfection,
iPSCs were exposed to N2 media containing 5 mL N2 Supplement-B (Stemcell Technologies 07156), 0.5mL 55mM β-mercaptoethanol (Life technologies 21985-023), 0.5mL primocin (Invivogen ant-pm-2), BDNF (10ng/mL, peprotech 450-02), NT-3 (10ng/mL, peprotech 450-03), laminin (200ng/mL, Sigma L2020), and doxycycline (2ug/mL, Sigma D3072) in DMEM / F12 (Gibco 11320-033) for 24 hours (DIV0). Cells were then exposed to puromycin (5ug/mL, Sigma P9620) for 24 hours in the same N2 media (DIV1). iNeurons were re-plated 24 hours later (DIV2) to experiment appropriate plates coated with Matrigel GFR (Corning 354230). Cells were washed 2x in PBS and lifted with StemPro accutase (Thermo Fisher Scientific A11105-01) for 5min at 37°C. Cells were spun down at 1000RPM for 5min at RT. Cells were resuspended and plated in iN media (Neurobasal-A media (Invitrogen A24775-01) with 5mM glucose (Sigma G5146), 10mM sodium pyruvate (Sigma P5280), glutamax (Life Technologies 35050-061), penicillin/streptomycin (Thermo Fisher Scientific 15140-148), BDNF and NT-3 (10ng/mL), and doxycycline (2ug/mL) through 9 days. 2µM Ara-C (Sigma C6645) was added on DIV3, and there was a full media exchange 24 hours later (DIV4). Doxycycline post DIV10 was discontinued for the rest of the 21-day differentiation.

4.2.3 CMP differentiation protocol
As described in (Paluru et al., 2014), differentiation began once iPSCs reached ~70% confluency. All three base media were supplemented with 2 mM glutamine, 50µg/ml ascorbic acid (Sigma), 150µg/ml transferrin (Roche
Diagnostics), and 400 µM monothioglycerol (MTG) (Sigma). The base media were RPMI (Invitrogen), StemPro-34 (SP-34) (Invitrogen), and serum free differentiation (SFD) media. The cultures were maintained at 37 °C in an environment of 5% CO2, 5% O2, and 90% N2. Days 0–1 RPMI with 5 ng/ml BMP4 and 50 ng/ml VEGF, and 1µM CHiR (Tocris 4423); Day 2 RPMI with 5 ng/ml BMP4, 50 ng/ml VEGF and 20 ng/ml bFGF; Day 3 SP34 with 5 ng/ml BMP4, 50 ng/ml VEGF and 20 ng/ml bFGF; Days 4–5SP34 with 15 ng/ml VEGF and 5 ng/ml bFGF; Day 6 SFD with 50 ng/ml VEGF, 100 ng/ml bFGF, 50 ng/ml SCF, and 25 ng/ml Flt3L; Days 7–9 SFD with 50 ng/ml VEGF, 100 ng/ml bFGF, 50 ng/ml SCF, 25 ng/ml Flt3L. Fresh media mixes (2 ml/well) were added each day. By day 6, media was increased to 4 ml/well. From days 7 to 9, single cells shed off the adherent layer into the medium and were collected. CMPs were frozen at 1-3 million cells per vial in 90% FBS and 10% DMSO.

4.2.4 iAstrocyte differentiation of iPSCs
iPSCs transfected with the NGN2 virus were put through the first two days of the iNeuron differentiation. On day 3, cells were exposed to Astrocyte differentiation media: N2 media (without BDNF, NT-3, laminin, and doxycycline), 10% FBS (Hyclone SH30071.03HI), B-27 with vitamin-A (Thermo Fisher Scientific 17504-044), FGF-2 (20 ng/mL, R&D Systems 233-FB-025) and EGF (20 ng/mL, R&D Systems 236-EG-200). After 30 days, EGF was removed and FGF2 reduced to 5 ng/mL. After 70 total days, cells were switched into Astrocyte Media
Experiments were performed after 90 total days of differentiation. After DIV90, iAstrocyte stocks were frozen down.

4.2.5 iAstrocyte passaging
After puromycin selection on DIV1 of NGN2 differentiation, NPCs are replated on DIV2 at 100,000 cells/cm². Once cells reach 95% confluency, iAstrocytes were washed 2x with PBS and then lifted with StemPro accutase (Thermo Fisher Scientific A11105-01) for 5 min at 37°C and then spun down at 1,000 rpm for 5 min and split 1:3. Cells were plated to plastic with no additional coating. This was consistent through the entire differentiation.

4.2.6 iAstrocyte cryopreservation
iAstrocytes were washed 2x with PBS and then lifted with StemPro accutase (Thermo Fisher Scientific A11105-01) for 5 min at 37°C and then spun down at 1,000 rpm for 5 min. Supernatant was aspirated and cell were resuspended in 90% Sciencell Astrocyte Medium and 10% DMSO and frozen at 1 million cells per vial.

4.2.7 iMicroglia differentiation of iPSCs
iPSCs were differentiated into common myeloid progenitors (CMPs) according to the published protocol (Paluru et al., 2014) by the Human Pluripotent Stem Cell Core (CHOP). CMPs were plated at 333K cells / well in a 24-well Cellbind plate (Corning 3337). CMPs were differentiated in iMg media (RPMI 1640 media (GE Healthcare Life Sciences SH30027.01) with 10% FBS (Hyclone SH30071.03HI), Recombinant Human IL-34 (100ng/mL, R&D systems 5265-IL-010), CSF-1
Recombinant Human Protein (25ng/mL, Thermo Fisher Scientific PHC9504), and Recombinant Human TGF-β1 (50ng/mL, Peprotech 100-21). Half media changes were performed every two days for 11 days.

4.2.8 Tri-culture combination
iNeurons were differentiated as described above and re-plated on DIV2 to Matrigel (Corning 354230) coated Nunc Lab-Tek II 8-well chamber slides (Thermo Fisher Scientific 62407-296) at 70k cells/well in iN media. On DIV5 of iNeuron differentiation, the iAstrocytes were added at 50k cells/well. On DIV7 of iNeuron differentiation, iMicroglia were added at 100k cell/well. Cultures were taken out to DIV21 of iNeuron differentiation. Of note, this preparation creates a “carpet-culture” of the three cell types that is roughly 50um thick.

4.2.9 Human astrocytes
Three separate donors for human astrocytes were obtained from Sciencell. Cells were plated on PLL-coated plates (Sigma Aldrich P6282) and grown for two weeks in Astrocyte media (Sciencell 1801) and then RNA was extracted.

4.2.10 iAstrocyte cytokine exposure
iAstrocytes in mono-culture were exposed to IL-1β (R&D Systems 201-LB-005) or IL-8 (R&D Systems 208-IL-010) at 10ng/mL or PBS vehicle control for 8 hours. Supernatants were collected and sent for cytokine analysis.

4.2.11 iAstrocyte Glutamate Uptake
iAst plated in 12-well dishes were placed in a 37°C waterbath and rinsed twice with 1ml of either sodium or choline containing buffer (5mM Tris Base, 10mM
HEPES, 2.5mM KCl, 1.2mM CaCl2 2H2O, 1. mM MgCl 6H2O, 1.2mM K2HPO4, 10mM Dextrose and 140mM NaCl or 140 mM choine chloride). The cells were then incubated with 0.5µM [3H]-Glutamate for 5 min in the absence or presence of 3mM TFB-TBOA (Tocris; cat #2532) for 5 minutes. Assays were ceased with the addition of 1 mL 4°C choline-containing buffer then solubilized in 1 mL of 0.1 N sodium hydroxide. An aliquot (500 µl) was combined with 5 ml of EcoLite (MP Biomedicals; cat #SKU 0188247501) and radioactivity was measured using a Beckman scintillation counter. Na+-dependent transport was calculated as the difference in radioactivity in the presence or absence of sodium and normalized to the amount of protein per well (Lowry protein assay).

4.2.12 Calcium wave propagation assay
iAstrocytes were tested for gap junction-dependent calcium wave propagation similarly to (Fujii, Maekawa, & Morita, 2017). iAstrocytes were grown on Matrigel (Corning 354230) (1:25 DMEM) coated glass coverslips at 90,000 cells/cm² and grown for one week in Astrocyte media (Sciencell 1801). Cells were washed 2x with RT HBSS (Corning MT21023CV) and then incubated in HBSS with 4µM Fluo-4 AM (Thermo-Fisher Scientific F14201) for 30min at 37° C. Cells were then washed 3x with HBSS and then left in in HBSS for imaging. Cells treated with carbenoxolone, a gap junction blocker, were incubated in HBSS with 100µM carbenoxolone for 10 minutes before imaging. All epifluorescence images were acquired with an exposure of 500ms under 10x magnification using a Nikon eclipse Ti-U microscope. The first baseline image was acquired prior to
mechanical stimulation of cells with a 3-5MOhm borosilicate pipette pulled with a Sutter P-97 Micropipette Puller. Pipettes were slowly advanced toward individual cells using a micromanipulator. t=0 images were captured immediately following visual confirmation of cell stimulation. Images were acquired every subsequent 15 seconds for 60 seconds.

4.2.13 Calcium wave analysis
The time-series of six images were combined into a virtual stack of spatially registered images using the ImageJ plugin Turboreg (rigid body translation). Both ΔFsti and ΔFprop were calculated using regional changes in fluorescence. ΔFstim was defined as change in total fluorescence intensity from baseline to t=0sec within the Center/Stimulation Region (within 100um of stimulation point). ΔFprop defined as change in total fluorescence intensity from t=0sec to t=15sec within the Surround Region (the adjacent outer-ring region extending 100um from the Center Region). Because mechanical stimulation of the cells with the micromanipulator increased risk of shifting the imaging plane, ΔFprop for the surrounding region was defined as F(t=15) – F(t=0).

To determine the rate of efflux from the initially stimulated cells, an average dF/F trace was calculated for each replicate trial. The technical replicates were then averaged to produce one dF/F trace per cell line. For each replicate experiment, individual dF/F cell traces (dF/F = (F-F0)/F0 = (F-F(t=0)) / F(t=0)) were calculated and averaged to determine each replicate’s average dF/F trace. ROIs for
individual cells were determined using ImageJ’s magic wand package for semi-automated segmentation of maximum Z-projections for each stack.

4.2.14 HIV infection
IMicroglia were differentiated to D11. On D11, they were exposed to 50ng/mL of JAGO strain HIV (UPenn Center of Aids Research; CFAR) for 24 hours. Media was fully exchange after 24 hours and collected. Full media exchanges occur every 3 days for 15 days. If antiretroviral treatment was used, it was started on day 6 of infection and added with each media exchange. Efavirenz (U.S. Pharmacopeia 1234103) was used at 20nM and Darunavir (Prezista TMC114) at 4.5µM.

4.2.15 Monocyte-derived macrophage differentiation
We receive donated buffy coat from New York Blood Center from three donors (D471, D446, D470). Buffy coat was diluted 1:1 with PBS (without Ca²⁺ and Mg²⁺) (Invitrogen 14190144). 15 mLs of Ficoll (Sigma Aldrich 26878-85-8) was added to 50mL conical tubes. 25mLs of Buffy Coat/PBS was slowly layered onto the Ficoll. Samples were spun at 1200 rpm for 45 minutes with no brake. The peripheral blood mononuclear cell (PBMC) layer was removed and placed into a new 50mL conical tube, and the volume was brought up to 50mL with PBS. The PBMCs are spun at 450Xg for 10 minutes. The supernatant was discarded, and the pellet resuspended in 10mLs of Red Blood Cell Lysis buffer (Sigma Aldrich 11814389001). Cells were shaking at RT for 10 minutes. Volume was brought up to 50mLs in PBS and spun at 450Xg for 10 minutes. The supernatant was
discarded, and the pellet was resuspended in DMEM with 10% FBS + Gentamicin (Thermo Fisher Scientific 15750060). The PBMCs were plated on 6-well tissue culture plate for 5 days. On day 5, a full media exchange was performed and added 10ng/mL Human GM-CSF (Gold Biotechnology 1120-03-20). A half media exchange was performed on day 7. At day 10, RNA was collected from macrophages.

4.2.16 RNA extraction
Cells were washed twice with RT PBS, then lifted with StemPro accutase (Thermo Fisher Scientific A11105-01) and spun down at 1,500 rpm for 5 min. Fresh cells were processed through Qiashredder (Qiagen 79654) and RNeasy mini kit (Qiagen 74104) and frozen at -80°C.

4.2.17 Immunofluorescence
Cells were washed twice with PBS, then fixed in 4% PFA (VWR TCP0018) for 15 min at RT. Cells were washed 3x in PBS for 5 min at RT before being stored in PBS at 4°C. Cells are blocked in 5% BSA (Sigma A9418) and 0.1% Triton X-100 (sigma X100) for 1 hr at RT. Sections use 0.3% Triton-X 100 in blocking buffer. Primary and secondary antibodies were diluted in blocking buffer. Cells were incubated in primary antibodies overnight at 4°C. Cells were washed 3x 5min each at RT in PBS-T (0.1% Tween20 (Sigma P9416). Secondary was performed at RT in the dark for 1 hour. Cells were washed 3x 5min each at RT in PBS-T, then mounted with Prolong gold antifade (Life Technologies P36930).
TMEM119 and DA1E IgG control antibodies required antigen retrieval. Before blocking, cells were heated to 100° C for 4 minutes in 10mM sodium citrate/0.05% tween20, pH 6, then incubated for 15 minutes at RT. After 15-minute incubation, cells were blocked as normal.

Antibodies used for immunofluorescence: Chicken anti-MAP2 (Abcam ab5392, RRID:AB_2138153, 1:500); Mouse anti-PSD-95 clone K28/43 (NeuroMab 75-028, RRID:AB_2292909, 1:500); Mouse anti-Synaptophysin clone SY38 (Millipore MAB5258-20UG, RRID:AB_95185, 1:250); Mouse anti-Nestin clone 10C2 (Millipore MAB5326, RRID:AB_11211837, 1:200); Rabbit anti-Thrombospondin-1 (Abcam ab85762, RRID:AB_10674322, 1:250); Mouse anti-Glutamine Synthetase (Millipore MAB302, RRID:AB_2110656, 1:500); Rabbit anti-SOX9 (Abcam ab185230, RRID:AB_2715497, 1:250); Rabbit anti-CX3CR1 (Abcam ab8021, RRID:AB_306203, 1:500); Rabbit anti-TMEM119 (Abcam ab185333, RRID:AB_2687894, 1:200); Rabbit anti-IBA1 (Wako 019-19741, RRID:AB_839504, 1:500); Rabbit anti-P2RY12 (Alomone Labs APR-020, RRID:AB_11121048, 1:100); Rat anti-LAMP1 (Abcam ab25245, RRID:AB_449893, 1:500); Mouse anti- HIV1 p24 [39/5.4A] (Abcam ab9071, RRID:AB_306981, 1:500); Rabbit anti-Human Nanog (Cell Signaling Technology 3580, RRID:AB_2150399, 1:800); Mouse anti-NCAM Clone 2-2b (Millipore MAB5324, RRID:AB_95211, 1:250); Rabbit anti-OCT-4 (Cell Signaling Technology 2750, RRID:AB_823583, 1:200); Rabbit anti-SOX2 (Millipore AB5603, RRID:AB_2286686, 1:100); Rabbit anti-CCR5 (Thermo Fisher Scientific pa5-29011, RRID:AB_2546487, 1:500); Mouse anti-GLAST (Miltenyi Biotec 130-
095-822, RRID:AB_10829302, 1:50); Mouse anti-GFAP (Sigma-Aldrich SAB1405864, RRID:AB_10739114, 1:10,000); Mouse anti-Beta-Actin (Cell Signaling Technology 3700, RRID:AB_2242334, 1:10,000); Rabbit anti-EAAC1 (Santa Cruz Biotechnology sc-25658, RRID:AB_2190727, 1:50); Rabbit GLT-1 (Jeff Rothstein Lab 1:5,000); Rabbit anti-DA1E IgG XP isotype control (Cell Signaling Technology 3900S, RRID: AB_1550038, 1:200); Goat anti-mouse IgG (H+L) Alexa Fluor 488 (Thermo Fisher Scientific A-11029, RRID:AB_138404, 1:500); Goat anti-rabbit IgG (H+L) Alexa Fluor 488 (Thermo Fisher Scientific A-11034, RRID:AB_2576217, 1:500); Goat anti-mouse IgG (H+L) Alexa Fluor 568 (Thermo Fisher Scientific A-11004, RRID:AB_2534072, 1:500); Goat anti-rabbit IgG (H+L) Alexa Fluor 568 (Thermo Fisher Scientific A-11036, RRID:AB_10563566, 1:500); Goat anti-rat IgG (H+L) Alexa Fluor 568 (Molecular Probes A-11077, RRID:AB_141874, 1:500); Goat anti-rat IgG (H+L) Alexa Fluor 680 (Molecular Probes A-21096, RRID:AB_141554, 1:500); Goat anti-Chicken IgY (H+L) DyLight 680 (Thermo Fisher Scientific SA5-10074, RRID:AB_2556654, 1:500)

4.2.18 iAstrocyte immunofluorescence counting
Images were obtained with a Nikon eclipse N1 scope equipped with LED-based epifluorescence. The optical fractionator workflow mode of Stereo Investigator 64 bit was then used to generate random areas of the wells to image. 5 images were obtained per well. Images were then transferred to image J where the channels were manually merged. After merging images, the “cell count” plug in of
imageJ is used to quantify the total number of DAPI (+) cells, and the number of co-labeled DAPI+ astrocyte marker(+) cells.

**4.2.19 Local Synapse Density**
Images were analyzed on ImageJ. The iMicroglia were outlined and had a 50µm radius circle drawn from the center of the cell with ROI manager. The total synapse density was measured using the analyze particles plugin. The plugin was used for the ROI of the cell and the surrounding circle. The area of the cell and the area of the particles were subtracted from the total area of the circle and the total particles. The remaining total area of the particles was divided by the remaining total area of the circle to calculate local synapse density.

**4.2.20 Bulk RNA-seq**
RNA was extracted using previously described with RNeasy mini kit (Qiagen 74104) and frozen at -80°C. Samples were sent to the Center for Applied Genomics (CAG) for sequencing. In short, the Illumina TruSeq Stranded Total RNA library kit (Illumina RS-122-2201) for RNA-seq was utilized for preparation of the libraries for sequencing. Libraries were produced using liquid handler automation with the PerkinElmer Sciclone instrument. This procedure started with a ribosomal RNA (rRNA) depletion step. The depletion step uses target-specific oligos with specialized rRNA removal beads to remove both cytoplasmic and mitochondrial rRNA from the total RNA. Following this purification, the RNA was fragmented using a brief, high-temperature incubation. The fragmented RNA was then reverse transcribed into first-strand cDNA using reverse
transcriptase and random primers. Second strand cDNA was generated using DNA Polymerase which was then used in a standard TruSeq Illumina-adapter based library preparation. Library preparation consisted of four main steps: unique adapter-indexes were ligated to the RNA fragments, AmpureXP bead purification occurred to remove small library fragments, the libraries were enriched and amplified using PCR, and the libraries underwent a final purification using AmpureXP beads. Upon completion, library quality was assessed using an automated electrophoresis instrument, the PerkinElmer Labchip GX Touch, and qPCR using the Kapa Library Quantification Kit and Viia7 real-time PCR instrument. Libraries were diluted to the appropriate sequencer loading concentration and pooled accordingly to allow for the desired sequencing depth. RNA libraries were sequenced in one lane of the Illumina HiSeq2500 sequencer using the High Output v4 chemistry and paired-end sequencing (2x100bp).

4.2.21 Single Cell RNA-seq cell preparation
Cells were incubated in 0.25% Trypsin+EDTA at 37°C for 8 minutes, put through a cell strainer, and spun down in ice-cold PBS at 4°C for 5 min at 1500 RPM. Cells were resuspended in ice-cold DPBS without Mg²⁺ and Ca²⁺+0.04% BSA. Up to 20,000 cells were sent for sequencing per sample. Samples were sent to CAG for sequencing.

4.2.22 Bulk RNA-seq analysis
RNA-seq reads were demultiplexed using the DRAGEN genome pipeline (Goyal et al., 2017). FASTQ files were aligned to hg19 reference using the STAR
(v.2.6.1) (Dobin et al., 2013) aligner with default settings. Generated BAM files were read into the R statistical computing environment. Gene counts were obtained using the GenomicAlignments package. Differential expression analysis was performed using the R/Bioconductor package DESeq2 which uses a negative binomial model (Love, Huber, & Anders, 2014). Analysis was performed using standard parameters with the independent filtering function enabled to filter genes with low mean normalized counts. The Benjamini-Hochberg adjustment was used to estimate the false discovery rate (Padj) and correct for multiple testing. Genes were then analyzed using the Ingenuity IPA software (QIAGEN Inc.). Additional published RNA-seq data was utilized for comparative analysis from project accession SRP092075 (Abud et al., 2017). Datasets were obtained and converted to fastq format using the Sequence Read Archive (SRA) tool provided by NCBI. Fastq-formatted data was analyzed similarly to the bulk RNA-seq samples using the DRAGEN pipeline and integrated into the experimental R data object.

4.2.23 Single Cell RNA-seq analysis
Next-generation sequencing libraries were prepared using the 10x Genomics Chromium Single Cell 3’ Reagent kit v2 per manufacturer’s instructions. Libraries were uniquely indexed using the Chromium i7 Sample Index Kit, pooled, and sequenced on an Illumina Hiseq sequencer in a paired-end, single indexing run. Sequencing for each library targeted 20,000 mean reads per cell. We had a mean of 39,227 reads per cell post-normalization with 2,165 median genes per
cell. Data was then processed using the Cellranger pipeline (10x genomics, v.3.0.2) for demultiplexing and alignment of sequencing reads to the GRCh38 transcriptome and creation of feature-barcode matrices. Individual single cell RNAseq libraries were aggregated using the cellranger aggr pipeline. Libraries were normalized for sequencing depth across all libraries during aggregation. Secondary analysis on the aggregated feature barcode matrix was performed using the Seurat package (v.3.0) within the R computing environment. Briefly, cells expressing less than 200 or more than 5000 genes were excluded from further analysis. Additionally, cells expressing >20% mitochondrial genes were excluded from the dataset. Log normalization and scaling of features in the dataset was performed prior to principal component dimensionality reduction, clustering, and visualization using tSNE. Cell types were identified using expression of canonical cell markers in microglia (AIF1, SPI1, CD4), neurons (MAP2, SYN1), and astrocytes (THBS1, SOX9). Differentially expressed genes and identification of cluster or cell type specific markers were identified using a wilcoxon rank sum test between two groups. P-value adjustment was performed using bonferroni correction based on total number of genes in the aggregated dataset. Genes were then analyzed using the Ingenuity IPA software (QIAGEN Inc.).

**4.2.24 qRT-PCR**

RNA was extracted by RNeasy mini kit (Qiagen 74104). cDNA was generated using SuperScript VILO Master mix (Thermo Fisher Scientific 11755050). RNA
expression was measured using Taqman probes (Thermo Fisher Scientific) for CX3CR1 (Hs01922583_s1), P2RY12 (Hs01881698_s1), TMEM119 (Hs01938722_u1), THBS1 (Hs00962908_m1), and GAPDH (Hs02786624_g1). 30ng of cDNA was used per well, with three technical replicates per probe per sample. qRT-PCR is run on an Applied Biosystems 7900HT Fast Real-Time PCR System. All expression levels were normalized to GAPDH expression.

4.2.25 Reverse transcriptase activity
10 µl per well of supernatant was placed into a 96-well microtiter plate to be analyzed for RT activity. 50 µl of RT cocktail was added per well into the 96-well microtiter plate. RT cocktail consists of: 50mM Tris (Amresco J837) pH 7.8, 75mM KCl (Ambion 9610), 2mM Dithiothreitol (DTT) (Sigma D0632), 5mM Magnesium Chloride (Ambion 9530G), 5ug/mL Polyadenylic acid (GE Healthcare 27-4110-01), 2.5ug/mL pd (t)12-18 (Oligo dT) (USB Corporation #19817), 0.05% NP-40 (Calbiochem, 492016), 10 µCi/ml Thymidine 5’-triphosphate, ALPHA-[32P] / [32P] TTP (Perkin Elmer BLU005A250MC ). Samples were incubated at 37° C overnight. 30 µl of RT reaction mixture was placed onto pre-marked DE81 paper (Whatman 3658915) and air dried for 15 min at RT. Paper was washed 4x, 5 min each with 2x SSC (Roche-Apply science 11 666 681 001) by submerging in a tray on a rotating platform. Paper was then washed 1x, 1 min in 100% ethanol and air dried in an oven at 80-100° C (25-30 min). Each paper sample was placed into a scintillation vial. 5 ml Betaflour (National Diagnostics LS-151)
was added to each scintillation vial. 32P was counted on a scintillation counter, yielding CPM.

4.2.26 PCR for CCR5Δ32 mutation
DNA was isolated with DNeasy blood and tissue kit (Qiagen 69504). DNA Oligos were generated by IDT. Forward primer: 5’ – CAAAAAGAAGGTCTTCATTACACC – 3’. Reverse primer: 5’ – CCTGTGCCTTTCTTTCTTTCATTTCG – 3’. Primers were reconstituted to 100uM. Mastermix consists of 10uL KAPA PCR buffer, 7uL H20, 1uL 10uM forward primer, 1uL 10uM reverse primer. 1uL DNA added separately. 1.5% agarose gel was run for 1 hour at 120v. The gel was imaged on a Biorad Universal Hood II Gel Doc System.

4.2.27 Western blot
iAst plated in 12-well dishes were rinsed twice with PBS containing 0.1 mM Ca2+ and 1.0 mM Mg2+ (PBS Ca2+/Mg2+) then lysed in 200ul of radioimmunoprecipitation (RIPA) buffer (100 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate containing protease inhibitors, including 1 mg/ml leupeptin, 250 mM phenylmethylsulfonyl fluoride, 1 mg/ml aprotinin, and 1 mM iodoacetamide) for 1 hour while rotating on a shaker at 4°C. Cortical and hippocampal tissue was harvested from adult C57BL/6 mice and solubilized in 5 volumes of homogenization buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% NP-40, 1% SDS, containing protease and phosphatase inhibitors, including 1 mg/ml leupeptin, 250 mM
phenylmethylsulfonyl fluoride, 1 mg/ml aprotinin, 1 mM iodoacetamide, 10 mM NaF, 30 mM Na pyrophosphate, 1 mM Na$_3$VO$_4$). All lysates were centrifuged at 17,000g for 20 min to remove cellular debris and nuclei. The supernatants were analyzed for total protein using the Pierce protein assay kit according to the manufacturer's instructions. Lysates are diluted 1:1 with 2X Laemmli buffer and either boiled at 95°C for 5 min (GFAP, Actin) or kept at 25°C for 45 minutes (GLAST, GLT-1, EAAC1). iAst lysates (20 ug) or cortical/hippocampal lysates (5 ug) were then resolved by SDS PAGE using 10% BioRad minigels and transferred to Immobilon-FL membranes (Millipore Cat# IPFL00010). Membranes were incubated with blocking buffer (1% non-fat dry milk in TBS-T) for 1 hour at 25°C prior to probing with primary antibodies diluted in blocking buffer overnight at 4°C: rabbit anti-GLT-1 (C-terminal directed 1:5,000; Rothstein et al 1994), mouse anti-GLAST (Miltenyi Biotec Cat# 130-095-822, 1:50), rabbit anti-EAAC1 (Santa Cruz Cat# SC-25658, 1:50), mouse anti-GFAP (Cell Signaling Cat#3670S, 1:1,000), and mouse anti-Actin (1:10,000 dilution, Cell Signaling, Cat# 3700S). Membranes were washed with blocking buffer 3 times for 10 min at 25°C. After the washes, membranes were probed with anti-mouse or anti-rabbit fluorescently conjugated secondary antibodies (LI-COR Biosystems, 1:10,000) for 45 minutes at 25°C. The membranes were washed 3 times for 10 minutes each then visualized using a LI-COR Odyssey.
4.2.28 Synaptophagocytosis analysis
Confocal images of microglia were analyzed in IMARIS software. IBA1 labeling for the microglia was surfaced, as well as synaptophysin staining. Total volume was taken for the microglia and the synaptophysin staining inside the cells. Total synaptophysin volume per cell was then divided by the cell volume defined by IBA1 labeling.

4.2.29 Cytokine analysis
Supernatants were tested for 6 analytes (TNFa, IL-6, IL-8, IL-10, IL-1b, IL-1a) on a custom Human magnetic Luminex plate (R&D systems LXSAHM; run by Penn Mental Health AIDS Research Center (PMHARC)). The plate was run by the Penn Mental Health Aids Research Center on a MAGPIX powered by Luminex XMAP technology.
4.3 Results

Figure 23: Generation and characterization of iMg

(A) Timeline for iMg differentiation and HIV infection.
(B) Immunostaining for microglia specific markers: CX3CR1, TMEM119, IBA1, and P2RY12. All cultures were stained for DAPI.

Figure 23: Generation and characterization of iMg

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4.3.1 iMicroglia exhibit similar gene and protein expression as other iPSC-derived microglia

To develop a tractable system for studying HAND in vitro, we adapted two previously published protocols to generate iMg from four separate iPSC lines (Abud et al., 2017; Paluru et al., 2014) (Figure 23A). First, iPSCs are differentiated into CD41+CD235+ common myeloid progenitors (CMPs) through a 9-day process (Paluru et al., 2014). Next, using a modified 11-day protocol (Abud et al., 2017) CMPs are differentiated into a highly pure population of ramified iMg that express CX3CR1, IBA1, TMEM119, and P2RY12 (Figures 24A, 23B, and 23C). Based on the Abud protocol, we used the small molecules IL-34 and CSF-1, as these are the ligands for CSF1R and are necessary for microglia development in vivo (Easley-Neal, Foreman, Sharma, Zarrin, & Weimer, 2019), and TGF-β1, as it helps induce an in vivo pattern of gene expression (Gosselin et
Fetal bovine serum was implemented to increase viability. iMg exhibited a 179-fold, 28-fold, and 11-fold increase in CX3CR1 (p<0.0001), P2RY12 (p<0.05), and TMEM119 (p<0.01) respectively over monocyte derived macrophages (MDMs) by qRT-PCR (Figures 23D-23F). Microglial identity was further confirmed by RNAseq (Figure 23G and 24B). Importantly, iMg also express CCR5 (S1C), one of the co-receptors necessary for HIV infection (Deng et al., 1996). Finally, we compared our iMg to human MDMs we generated as well as datasets from primary human adult and fetal microglia, iPSCs, induced hematopoietic progenitor cells, and iMicroglia from Abud and colleagues (Abud et al., 2017). PCA revealed closer clustering of our iMg (pink) to primary human microglia (blue) than previously published iPSC-derived microglia (light orange) (Figure 23H).
Figure 24: Morphology and comparative RNA expression of iMG to MDMs and CCR5Δ32 mutation

(A) Brightfield images of iMg differentiation, depicting ramification by D11.
(B) MA plot of bulk RNAseq data comparing iMg (baseline) to MDMs. n=3 Benjamini-Hochberg FDR-0.05.
(C) Immunostaining showing CCR5 expression (green) on D11 iMg. scale bar represents 25um.
(D) PCR of CCR5Δ32 shows WT4 is heterozygous for the mutation.
(E) Infected WT4 has dramatically less raw reads of P24 from Bulk RNAseq compared to infected WT6 and WT10 iMg.
Figure 25: HIV-infected iMicrogla produce an inflammatory response, and respond to EFZ

(A) Immunostaining showing reduced percentage of P24+(red) IBA1+(green) iMg in HIV-infected mono-cultures + EFZ treatment compared to infected cultures with no EFZ treatment.
(B) Percentage of P24+ cells in mono-culture iMg at D15 for infected and Inf+EFZ conditions. n=4 infections, error bars represent SEM.
(C) Percent of P24+ single-nucleated and multi-nucleated iMg in mono-culture for infected and Inf+EFZ conditions. n=4 infections.
(D) Percent of P24(-) single-nucleated and multi-nucleated iMg in mono-culture for Inf and Inf+EFZ conditions. n=4 infections.
(E) Reverse transcriptase activity of Uninf, Inf, and Inf+EFZ (20nM) iMg show productive infection and response to EFZ. n=3 infections, one-way ANOVA, Dunnett’s post hoc analysis, *p<0.05; ****p<0.0001, error bars represent SEM.
(F-I) Cytokine analysis of Infected iMg mono-culture displays increase in IL-1b (F), IL-8 (G), TNFa (H), and IL-1a (I) production in the infected iMg. n=3 infections, one-way ANOVA, Dunnett’s post hoc analysis, *p<0.05; **p<0.01, error bars represent SEM.
(Ryan SK, Sade-Williams K)

4.3.2 iMg Are productively infected with HIV and respond to multiple antiretrovirals

To establish the effect of HIV infection and ART on microglia, we first used iMg mono-culture. We first determined infectivity of the iMg by exposing cultures to 50ng/mL of the CSF-derived, R5-tropic JAGO strain of HIV (Chen et al., 2002). After a 15-day infection, a time point based on prior studies of MDMs (O'Donnell et al., 2006), 94.5 ± 5.5% of the iMg were positive for the HIV capsid protein P24 and exhibited vast multinucleation (Figures 25A and 25B). Most (89.1 ± 3.8%) P24(+) iMg were multi-nucleated (Figure 25C), while no P24(-) iMg were multi-nucleated (Figure 25D), showing that multi-nucleation is exclusively associated
with infection. Reverse transcriptase activity showed peak viral production occurred near day 15 (Figure 25E).

We initially infected iMg from iPSCs of four individuals. Remarkably, the iMg of one individual showed limited infection (Figure 24E). Subsequent genotyping revealed heterozygosity for the CCR5Δ32 mutation, known to reduce infectivity by HIV (R. Liu et al., 1996) (Figure 24D). This line was thus excluded from subsequent studies, but this example demonstrates the validity of our system to model known regulators of human infection. To our knowledge, this the first report in which iPSC-derived microglia are infected with HIV.

To determine if ART can suppress infection in the iMg, we examined the effects of the antiretroviral drug efavirenz (EFZ), a non-nucleoside reverse transcriptase inhibitor (De Clercq, 2004). EFZ remains a first line drug in many parts of the world (Best & Goicoechea, 2008; Taramasso et al., 2018). Because EFZ blocks HIV reverse transcription, it had an advantage in allowing the study of non-productively infected, HIV-exposed cells alongside productively infected cells, a scenario thought to occur in brains of ART-treated HIV+ people. At day 15, EFZ reduced the infection rate by two-thirds to 29.2 ± 6.3% (Figures 25A and 25B). Of those iMg infected in the EFZ condition, rates of multinucleation were over 90% and similar to the Inf condition (Figure 25C), and 100% of P24(-) iMg in the Inf+EFZ culture were single-nucleated (Figure 25D). Reverse transcriptase activity was severely reduced with EFZ treatment (Figure 25E). The combined impaired reverse transcriptase and reduced percentage of P24(+) cells shows
that EFZ effectively suppressed new infection, allowing us to study both infected and uninfected iMg in the same culture.

4.3.3 Infected iMg produced proinflammatory cytokines at peak infection, which is tempered by EFZ treatment

HIV infection in the CNS leads to changes in cytokine profiles (Ginsberg et al., 2018; Tatro, Soontornniyomkij, Letendre, & Achim, 2014), as such, we quantified the changes in production of relevant cytokines in iMg over the course of infection ± EFZ. Infection led to increased production of several pro-inflammatory cytokines, specifically IL-1b (p<0.01), IL-1a (p<0.05), TNFa (p<0.05), and most prominently, IL-8 (p<0.01) (Figures 25F-25I). However, the two other cytokines tested: IL-6 and IL-10, did not change across any condition (Figures 26A and 26B). It was expected that the anti-inflammatory IL-10 would not increase, but surprising that IL-6 did not increase (Shah et al., 2011).

Infected iMg cultures exposed to EFZ had less production of IL-1b, IL-8, TNFa, and IL-1a (Figures 25F-25I), suggesting a reduced inflammatory reaction, which recapitulates what is seen in ART-treated patients. Uninf, Uninf+EFZ and the DMSO vehicle control did not elucidate a cytokine reaction across the six cytokines tested (Figures 26C-26G).
Figure 26: Additional cytokine expression during infection and with vehicle control

(A&B) IL-6 (A) and IL-10 (B) production in mono-culture did not change for any of the four conditions. n=3 differentiations (n=4 Uninf and Uninf+EFZ, n=3 Inf and Inf+EFZ), one-way ANOVA, Dunnett’s post hoc analysis, error bars represent SEM.

(C-F) cytokine production in Uninf and Uninf+EFZ mono-cultures. IL-8 (C), TNFa (D), IL-1a (E), and IL-1b (F) production in mono-culture did not change.
for Uninf or Uninf+EFZ. n=4 differentiations, one-way ANOVA, Dunnett’s post hoc analysis, error bars represent SEM.

(G) Uninf+ Veh. had minimal but significant increases in IL-8 and IL-1a production at D3, but no increase at D12 where infected cultures had significant increases. n=3 differentiations, one-way ANOVA, Dunnett’s post hoc analysis, ***p<0.001, error bars represent SEM.

(H&I) No change in tri-culture IL-10 (H) for any of the four conditions, but IL-6 (I) production increased over time for Uninf, and. n=3 differentiations, two-way ANOVA, Tukey’s post hoc analysis, **p<0.01; ****p<0.0001, error bars represent SEM.

(Ryan SK)

4.3.4 Infected iMg have impaired cell cycle regulation and DNA repair and increased expression of inflammatory genes

To investigate overall gene expression changes during infection, we performed bulk RNAseq on three iMg lines ± HIV infection, which revealed significant changes in cell cycle regulation and DNA repair (Figure 27A), consistent with our observation that HIV infection of iMg results in multinucleation. While no inflammatory pathways were identified by Ingenuity Pathway Analysis (IPA), many inflammatory genes involved in the complement system, NF-κB signaling, and TNFα signaling were significantly upregulated, as well as IL1b, CCL8, and FOS (Figure 27C).

Collectively, these data show that the iMg exhibit an inflammatory response similar to that seen in vivo with increased production of IL-8, IL-1b, IL-1a, and TNFα. This inflammatory response is strongly attenuated with Efavirenz treatment. RNAseq analysis revealed changes in cell cycle and inflammation between uninfected and infected iMg, as expected.
Figure 27: Bulk RNAseq analysis of uninfected versus infected iMg in mono-culture and IL-1α production in iAst

(A) Ingenuity Pathway Analysis of Uninfected v Infected iMg bulk RNAseq. n=3 cell lines, Fisher’s exact <0.05. Benjamini-Hochberg FDR 0.05.

(B) iAst produced IL-1α in response to 8hr exposure to IL-1β (10ng/mL), but not IL-8 (10ng/mL). n=3 cell lines, one-way ANOVA, Dunnett’s post hoc analysis, ****p<0.0001, error bars represent SEM.

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(C) Select inflammation related genes upregulated in iMg+HIV bulk RNA-seq. Log2fold change at least 1 and FDR <0.05.
(Ryan SK, Gonzalez MV)
Figure 28: Characterization of iAstS
(A) By day 2 of the NGN2 differentiation (post puromycin selection), cells express have lost pluripotency markers (Nanog, OCT4, and Sox2) and begin to express neural progenitor markers Nestin and NCAM, as well as, the astrocyte marker Sox9. Scale bar represents 25µm.

(B-D) Western blot showing iAst do not express glutamate transporter GLT-1 (B), GLAST (C), or GFAP (D) in mono-culture.

(E) scRNAseq analysis shows that, in tri-culture, iAst express GLT-1. iAst n=4,763; iMg n=4,485. two-tailed t test, ****p<0.0001, error bars represent SEM.

(F) Glutamate uptake assay showing uptake by three iAst lines and impaired uptake with exposure to glutamate transporter inhibitor TFB-TBOA. n=3 independent experiments for each cell line. Two-tailed paired t test. *p<0.05.

(G) Representative images of trans-cellular calcium propagation in iAst mono-culture ± 100µM carbenoxolone (CBX). ΔFstim is the change in fluorescence from baseline to stimulation within the central region. Dotted circle denotes initial stimulation area. ΔFprop is the change in the surrounding region’s fluorescence between 0 and 15 seconds. Center region radius = 100µm; Surround region radius = 200 µm. Scale bar represents 50µm.

(H) Representative ΔFstim and ΔFprop images, along with summary graphs that include all three lines. CBX does not significantly influence connexin-independent ΔFstim, while CBX does significantly reduce fluorescence propagation (ΔFprop). RFU=relative fluorescent units. n=3 cell lines (3 technical replicates per line). Two-tailed paired t test. n.s.= not significant, *p<0.05.

(I) Change in dF/F of individual cells that were mechanically stimulated. CBX significantly decreases decay rate of dF/F. n=3 cell lines (3 technical replicates per line). Two-way ANOVA, Sidak’s post hoc analysis, error bars represent SEM. *p<0.05.

(Ryan SK, Sotuyo NP, Mironets E)

4.3.5 Generation of iPSC derived tri-cultures of iNeurons, iAstrocytes, and iMicroglia

iPSC-derived neurons (iNrns) were generated by an established protocol that generates a homogenous population of glutamatergic, forebrain-like excitatory neurons (Yingsha Zhang et al., 2013). iPSC-derived astrocytes (iAsts)
were generated by brief exposure of IPSCs to the Ngn2 transcription factor used to drive iNrn differentiation, and that in vivo is expressed by cortical progenitors prior to their conversion from neurogenesis to gliogenesis. We noticed that after 2 days of Ngn2 exposure cells express the neural progenitor markers Nestin and NCAM, and the astrocyte marker SOX9 (Kang et al., 2012) (Figure 28A). Given that the NGN2 protocol produces a homogenous population of neurons, we posited that shifting the differentiation at the neural progenitor stage would yield a relatively homogenous population of iAsts. We thus shifted the differentiation to astrocytes by removing the NGN2-inducing agent doxycycline at day 2, while promoting astrocytic differentiation and proliferation with FGF-2, EGF, and FBS (Michler-Stuke, Wolff, & Bottenstein, 1984). After 70 days the iAsts were switched to Sciencell Astrocyte Media (Figure 29A). After 90 days, RNAseq revealed similar overall gene expression between iAsts and fetal human astrocytes (Hu Ast), including several key astrocyte genes (Figures 29B and 29F). qRT-PCR validation of THBS1 confirmed similar expression Hu Ast and iAst (Figure 29C). We confirmed several of these genes at the protein level, including Nestin, Glutamine Synthetase, THBS1, an important protein in early synaptogenesis (Christopherson et al., 2005), and SOX9, revealing a relatively homogenous population (Figures 29D and 29E). The iAsts exhibited modest glutamate uptake and propagated Ca\textsuperscript{2+} waves in a gap junction-dependent manner, demonstrated by halted Ca\textsuperscript{2+} propagation with 100µM carbenoxolone, a gap junction blocker (Figure 28F-28I). While in mono-culture, the iAsts did not detectably express GFAP, GLT-1, or GLAST (Figures 28B-28D), in tri-culture,
scRNAseq showed expression of $GLT-1$ in iAst (Figure 28E). As neuronal activity regulates GLT-1 in astrocytes (Swanson et al., 1997), iAst have a more $in vivo$-like phenotype when in the more physiologically relevant tri-culture.
We also exposed the iAst in mono-culture to the most highly expressed cytokines in the HIV-infected iMg, to determine whether the cytokines produced by infected iMg can elicit an inflammatory response in the iAst. We exposed the iAst to IL-1b and IL-8 at 10ng/mL for eight hours and then analyzed the supernatants on the same six-cytokine panel. We found that, of the six cytokines tested, the iAst produced increased amounts of IL-1a (Figure 27B), suggesting that the iMicroglia can elicit an inflammatory response in the iAst. In addition, it is interesting that IL-1a was released given its role in producing A1 astrocytes (Liddelow et al., 2017).
We next sought to study iMg in an *in vitro* setting amenable to the study of synaptic phagocytosis and the influences of HIV and ART on gene expression in forebrain cells. To accomplish this we independently generated neurons, astrocyte, and microglia-like cells, then placed them in mixed cultures. For the current study, analysis was performed 14 days after all three cell types are combined (Figure 29G), as our intention was to focus on the acute phase of exposure of forebrain-like neurons, astrocytes and microglia to productively HIV infected iMg. Having validated mono-cultures and assembled tri-cultures, we next used scRNAseq to investigate cell-type specific gene expression changes during infection ± EFZ.
Figure 30: scRNAseq identified each of the three cell types in all four conditions
(A) Timeline from start of CMP differentiation through tri-culture for the four conditions.
(B) t-SNE of unbiased clustering of combined scRNAseq from all four conditions.
(C-E) expression patterns of cell type specific markers for microglia (C), neurons (D), and astrocytes (E).
(F) t-SNE clustering by cell type based on cell type specific marker expression. One cluster did not align with any of the three cell types by the expression patterns chosen in (C-E).
(G) Heat map of top 20 genes expressed in each cell type reveals the undesignated cluster of cells to be similar to iAst expression profile, suggesting they may be immature iAst.
(Ryan SK, Gonzalez MV)

4.3.6 scRNAseq identified each of the three cell types in all four conditions

To create the tri-culture, we began the iNrn differentiation, added the iAst post-puromycin selection at D5 of the iNrn differentiation, and added the iMg at D7 of the iNrn differentiation, so that the iAst had time to acclimate before addition of HIV-infected iMg. The tri-culture was maintained for an additional 14 days, to D21 of the iNrn differentiation in NBM/B27 with NT-3 and BDNF as described before (Yingsha Zhang et al., 2013). To assay gene expression changes in each of the three cell types during HIV infection ± EFZ, we conducted scRNAseq on under four conditions: Uninf, Inf, Inf+EFZ, and Uninf+EFZ (Figure 30A). Cells were sequenced from the Uninf (n=6,564), Inf (n=7,431), Inf+EFZ (n=7,111), and Uninf+ EFZ (n=10,071) conditions, with comparable numbers of each cell type per condition (Figure 31D). All conditions were then aggregated and initially analyzed through the Cellranger pipeline (10x Genomics, v.3.0.1). Secondary analysis was performed using the Seurat package in R. We
generated 16 unbiased clusters (Figure 4B). First, we separated the clusters by cell type, then broke down each cell cluster by condition. We assigned clusters to one of the three cell types by expression of several key genes: iMg by expression of \textit{IBA1}, \textit{PU.1}, and \textit{CD4}; iNrn by expression of \textit{MAP2} and \textit{SYN1}; and iAst by expression of \textit{THBS1} and \textit{SOX9} (Figures 30C-30E). There were four clusters (cluster 5, 10, 12, and 13) that did not fit into any of the three cell types by expression of the chosen markers (Figures 30B and 30F). To determine what the fourth cell type might represent, we examined the expression of the top 20 genes in each of the 4 cell types (Figure 30G). The gene expression pattern of the undesignated cells best matched the iAst, but scRNAseq did not capture expression of \textit{THBS1} or \textit{SOX9}, suggesting these cells represent less mature versions of the iAsts. Hence, they were excluded from further analysis (Figure 31A). We then separated each cell type by condition (Figure 31B). We examined expression of genes related to inflammation and found the largest change in the iMg among the three cell types (Figure 31C).
Figure 31: iMg exhibit the largest inflammatory response by gene expression among the three cell types in tri-culture.

(A) t-SNE plot of all cells from all conditions excluding the undesignated cluster.
(B) t-SNE from (A) broken down by condition.
(C) Heatmap of inflammation, DNA accessibility, mitochondria, and translation/UPR related genes in iAst, iNrn, and iMg in all four conditions: Uninf, Uninf+EFZ, Inf, Inf+EFZ.
(Ryan SK, Gonzalez MV)
Figure 32: iMg activate RhoGDI and CD40 in response to HIV infection with EFZ treatment.

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Genes Dysregulated</th>
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<tbody>
<tr>
<td>RhoGDI Signaling</td>
<td>RND3, CD44, ACTG1, PP1R12A</td>
</tr>
<tr>
<td>CD40 Signaling</td>
<td>NFKBIA, ICAM1, TNFAIP3, FOS</td>
</tr>
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(A) t-SNE plot of Uninf and Inf conditions.
(B) Ingenuity pathway analysis of iMg between Uninf and Inf conditions. Uninf is baseline. Benjamini-Hochberg FDR 0.05, Fisher’s exact <0.05, z-score cutoff ± 0.5.
(C) t-SNE plot of Uninf and Inf+EFZ conditions.
(D) Ingenuity pathway analysis of iMg between Uninf and Inf+EFZ conditions. Uninf is baseline. Benjamini-Hochberg FDR 0.05, Fisher’s exact <0.05, z-score cutoff ± 0.5.
(E) Specific genes dysregulated that are involved in the RhoGDI and CD40 pathways in Inf+EFZ iMg compared to Uninf iMg. Red genes are downregulated. Green genes are upregulated.
(Ryan SK, Gonzalez MV)

4.3.7 Inflammatory pathways and EIF2 signaling were dysregulated among all three cell types during infection, but iMg were most affected

To understand the gene expression changes among the three cell types during HIV infection ± EFZ, we first compared the Inf condition to Uninf. Several inflammatory pathways were significantly activated in Inf iMg compared to Uninf iMg, including Il-8 and NF-κB signaling. One of the top pathways dysregulated in Inf iMg compared to Uninf was the EIF2 pathway (Figure 32B). EIF2 signaling is involved in the UPR and, more broadly, the integrated stress response (ISR) (Janssens et al., 2014). The EIF2 pathway was not only dysregulated in iMg, but also was consistently increased in the iAst and iNrn (Figures 33A and 33B). However, only iMg had increased expression of ATF4 mRNA, the downstream transcription factor of the PERK arm of the ISR, which leads to increased cytokine production (Masuda, Miyazaki-Anzai, Levi, Ting, & Miyazaki, 2013) in Uninf v Inf (Figure 31C). Previously, we have shown that the ISR, particularly the PERK arm, is activated in neurons and astrocytes from human brain samples
4.3.8 Inf+EFZ caused distinct increased activation of RhoGDI and CD40 pathways

We next compared Uninf to Inf+EFZ, expecting to see a dampened immune response compared to Inf. Many of the top affected pathways in Inf iMg were related to inflammation, and there is a stark difference between Inf iMg and Inf+EFZ iMg (Figures 34A and 34B). However, the Inf+EFZ had a much milder inflammatory reaction, where RhoGDI and CD40 signaling were the only upregulated pathways compared to Uninf iMg (Figures 32D and 32E). RhoGDI negatively regulates Rac, which functions in multiple inflammation pathways (Wilkinson & Landreth, 2006), and CD40 activates NF-κB signaling (D’Aversa, Eugenin, & Berman, 2008; Homig-Holzel et al., 2008). A milder inflammatory response corroborates well with lesser disease severity seen in HAND patients who are taking ART (Saylor et al., 2016). Interestingly, EIF2 signaling was only activated in the iNrns and sirtuin signaling was inhibited in all three cell types in Inf+EFZ (Figures 32D, 33C, and 33D).
The scRNAseq data suggest the microglia were most affected by the infection ± EFZ, with major changes to EIF2 signaling, inflammatory, oxidative damage, and phagocytic gene pathways. However, the distinct activation of
RhoGDI and CD40 in the Inf+EFZ condition suggests the combination of Inf+EFZ creates a unique effect not seen with infection or EFZ alone.

4.3.9 Inf+EFZ had an attenuated, but distinct inflammatory response compared to Inf and Uninf+EFZ
Inf+EFZ treatment yields a distinct inflammatory response in iMg compared to Inf and Uninf+EFZ

(A) t-SNE plot of Inf+EFZ and infected conditions. 
(B) Ingenuity pathway analysis of iMg between Inf+EFZ and infected conditions. Inf+EFZ is baseline. Benjamini-Hochberg FDR 0.05, Fisher's exact <0.05, z-score cutoff ± 0.5.
(C) t-SNE plot of Uninf+EFZ and Inf+EFZ conditions. 
(D) Ingenuity pathway analysis of iMg between Uninf+EFZ and Inf+EFZ conditions. Uninf+EFZ is baseline. Benjamini-Hochberg FDR 0.05, Fisher's exact <0.05, z-score cutoff ± 0.5.
(E) Specific genes dysregulated that are involved in the RhoGDI and CD40 pathways in Inf+EFZ iMg compared to Uninf+EFZ iMg. Red genes are downregulated. Green genes are upregulated. 
(Ryan SK, Gonzalez MV)

Inf iMg had increased activity in several inflammatory pathways including IL-6 signaling, neuroinflammation signaling, and Fcγ receptor-mediated phagocytosis compared to Inf+EFZ, suggesting the Inf+EFZ had an overall lower immune response. However, the EIF2 signaling pathway, part of the ISR and UPR, was higher in Inf+EFZ iMg compared to Inf and the NRF2-mediated oxidative stress response pathway was higher in the Inf+EFZ iNrls than Inf iNrls (Figures 34B and 35B). This became more complex as EIF2 signaling was increased in the Inf iAst and iNrls compared to Inf+EFZ (Figures 35A and 35B). These results suggest a mitigated, but distinct inflammatory response in Inf+EFZ compared to infection alone, and that each cell type may respond differently to EFZ.
Figure 35: iAst and iNrn Ingenuity pathway analysis for Inf+EFZ v Inf

(A&B) Top affected pathways from Ingenuity pathway analysis of iAst (A) and iNrn (B) in the Infected + EFZ versus infected conditions. Infected + EFZ is baseline. Benjamini-Hochberg FDR 0.05, Fisher's exact <0.05, z-score cutoff ± 0.5.
(Ryan SK, Gonzalez MV)

We next compared Inf+EFZ to Uninf+EFZ (Figure 35A). EIF2 signaling was increased in the Inf+EFZ iMg (Figure 34D), similar to the Inf and Inf+EFZ comparison (Figure 34B). However, inflammatory pathways including neuroinflammation and ROS/iNOS signaling were lower in Inf+EFZ compared to Uninf+EFZ iMg (Figure 34D) Inf+EFZ iMg again had increased activation of the CD40 and RhoGDI pathways (Figures 34D and 34E) compared to Uninf+EFZ, suggesting a distinct inflammatory response with the combination of infection and EFZ treatment. iAst had activation of the neuroinflammation and RhoGDI signaling pathways in Inf+EFZ compared to Uninf+EFZ (Figure 37E). These results show a stark difference in responses to EFZ treatment alone and EFZ
treatment with infection, suggesting combinatorial and probably interacting effects of infection and EFZ treatment.
Figure 36: EFZ treatment alone elicits comparable response as infection

(a) t-SNE plot of Uninf and Uninf+EFZ conditions. (b) Ingenuity pathway analysis of iMg between Uninf and Uninf+EFZ conditions. Uninf is baseline. Benjamini-Hochberg FDR 0.05, Fisher’s exact <0.05, z-score cutoff ± 0.5. (c) t-SNE plot of Uninf+EFZ and Inf conditions. (d) Ingenuity pathway analysis of iMg between Uninf+EFZ and Inf conditions. Uninf+EFZ is baseline. Benjamini-Hochberg FDR 0.05, Fisher’s exact <0.05, z-score cutoff ± 0.5.

(Ryan SK, Gonzalez MV)

4.3.10 Uninf+EFZ creates an inflammatory response similar to Inf

Inf+EFZ had a starkly different response than Uninf+EFZ. So, we further investigated the effect of EFZ treatment alone compared to Uninf and Inf treatments. ART can be used as a preemptive treatment against HIV infection; however, it has various inflammatory and neurotoxic side effects (Cagla Akay et al., 2014; Robertson et al., 2012). How ART affects different cell types in co-culture has not been explored. We compared Uninf+EFZ to the Uninf (Figures 36A, 36B, 37A, and 37B) and Inf (Figures 36C, 36D, 37C, and 37D). Remarkably, EFZ treatment alone created distinct changes in immune-related signaling pathways in the iMg (Figure 36B). We found a surprisingly similar gene expression profile to the Inf condition, especially in the iMg and iAst (Figure 36D and 37C). The pairwise comparison of Uninf and Uninf+EFZ revealed multiple inflammatory pathways increased in Uninf+EFZ iMg, with neuroinflammation signaling as the most activated pathway (Figure 36A and 36B). Several of the other pathways activated, including IL-6 and IL-8 signaling, were also found in the Uninf v Inf comparison (Figure 32B). EIF2 signaling was again increased in Uninf+EFZ iAst and iNrns compared to Uninf (Figure 37A and 37B). These data
suggest Uninf+EFZ elicits a similar, but not identical inflammatory response as Inf.

We found iMg had higher activation of neuroinflammation signaling and NRF2-mediated oxidative stress response in Inf compared to Uninf+EFZ (Figures 36C and 36D). Inf iNrs had impaired sirtuin signaling and higher oxidative phosphorylation activation compared to Uninf+EFZ iNrs; however, we found no statistically significant changes in any canonical pathways in iAst between the conditions (Figures 37C and 37D), suggesting an inflammatory response by iMg and change in mitochondria function and cellular stress in the iNrs.
Figure 37: iAst and iNrn top canonical pathways changed in Uninf v Uninf+EFZ, Uninf+EFZ v Inf, and Uninf+EFZ v Inf+EFZ
(A&B) Top affected pathways from Ingenuity pathway analysis of iAst (A) and iNrn (B) in the uninfected versus uninfected + EFZ conditions. Uninfected is baseline. Benjamini-Hochberg FDR 0.05, Fisher’s exact <0.05, z-score cutoff ± 0.5.

(C&D) Top affected pathways from Ingenuity pathway analysis of iAst (C) and iNrn (C) in the uninfected + EFZ versus infected conditions. Uninfected + EFZ is baseline. Benjamini-Hochberg FDR 0.05, Fisher’s exact <0.05, z-score cutoff ± 0.5.

(E&F) Top affected pathways from Ingenuity pathway analysis of iAst (E) and iNrn (F) in the Uninfected + EFZ versus infected + EFZ conditions. Uninfected + EFZ is baseline. Benjamini-Hochberg FDR 0.05, Fisher’s exact <0.05, z-score cutoff ± 0.5.

(Ryan SK, Gonzalez MV)

Infection, EFZ treatment alone, and infection with EFZ treatment all induced distinct responses not only across conditions, but also across cell types within conditions. These data reveal a potential downside to pre-emptive ART use and suggest that the inflammatory response from Infected to Inf+EFZ to Uninf+EFZ is not a linear regression as one might expect, but rather three distinct responses. We further investigated these gene pathway changes by testing for cytokine production and phagocytic abilities.
Figure 38: HIV-infected iMicroglia have reduced synaptophagocytosis, which is ameliorated by EFZ treatment

(A&B) Immunostaining (A) and surface reconstruction of a side view (B) of iMg in an iNrn co-culture displaying synaptic phagocytosis by co-localization of synaptophysin+ (red) puncta within LAMP1+ (blue) lysosomes in IBA1+ (green) iMg.

(C) Giant multi-nucleated IBA1+ (green) iMg potentially interacting with synaptophysin+ (red) synapses on MAP2+ (yellow) dendrites.

(D) Multi-nucleated iMg, but not MAP2+ (green) iNrn or iAst, are P24+ (red) in the tri-culture after 14 days.

(E) Representative surface reconstructions of synaptophysin signal (red) within IBA1+ (green) iMg in the Uninf, Uninf+EFZ, and Inf tri-cultures. SN—single nucleated. MN—multinucleated. Scale bar represents 5µm.

(F) Synaptophagy is significantly decreased in infected iMg ± EFZ and uninfected iMg + EFZ compared to control. n=4 (Uninf=4, Uninf+EFZ=4, Inf=6, Inf+EFZ=6) differentiations, one-way ANOVA, Dunnett’s post hoc analysis, *p<0.05, error bars represent SEM.

(Ryan SK)
4.3.11 iMg synaptophagocytosis is impaired in Inf, Inf+EFZ iMg, and Uninf+EFZ iMg

To further explore the differences in iMg responses to infection ± EFZ and EFZ treatment alone, we interrogated the ability of iMg to phagocytose synapses. Fcγ receptor mediated phagocytosis pathway was activated in the Inf iMg as well as the Uninf+EFZ iMg compared to Uninf iMg (Figures 32B and 36B). Many antiretrovirals, including Efavirenz, have been reported to produce neurotoxic effects (Ciccarelli et al., 2011; Robertson et al., 2012) and to reduce phagocytosis (Giunta et al., 2011). In addition, HIV-infected macrophages and uninfected macrophages exposed to HIV display decreased phagocytic capabilities, specifically through the Fcγ receptor-mediated pathway, due to Nef and Tat inhibiting endocytosis (Debaisieux et al., 2015; Giunta et al., 2008; Mazzolini et al., 2010). However, human microglia synaptophagocytosis in the context of HIV infection has not been tested.

In tri-cultures evaluated 14 days after addition of the iMg, we found colocalization of SYN1 in the LAMP1+ lysosomes of the iMg (Figure 38A and 38B), suggesting that the iMg phagocytize synaptic material. We confirmed that iMg are in contact with iNrns (Figure 38C), and importantly, only the iMg are infected as only they, and not the iNrns (Figure 38D) or iAsts, show P24 immunofluorescence. Additionally, 1 out of 1,208 iAst in the Inf condition had reads for p24 from the scRNAseq. These results suggest that it is unlikely that astrocytes are productively infected but may internalize HIV particles from the infected microglia at very low levels. We were also able to delineate infected from
uninfected iMg in the Inf+EFZ condition by multi-nucleation, which is present in nearly all P24+ iMg (Figures 25A and 39B).

The Inf iMg phagocytosed 70.9% less synapses than to Uninf iMg (p<0.01). In addition, in the Inf+EFZ condition, the infected (multinucleated) had 68.2% reduced phagocytosis (p<0.01); however, the uninfected (single-nucleated) iMg in the Inf+EFZ condition showed trends towards reduction in synaptophagy but was not statistically significant (p=0.01). Lastly, Uninf+EFZ condition iMg phagocytosed 57.1% less than Uninf iMg (p<0.05) (Figure 38E and 38F). Multiple factors from the virus itself, the immune response, and the effects of antiretrovirals are acting on the iMg during HIV infection. All these factors may play a role in reducing synaptic phagocytosis and warrant future study.
Figure 39: No change in synaptic density local to microglia or throughout the culture

(A) Cartoon representation of synaptic density analysis
(B) Immunostaining of IBA1+ (green) iMg in Uninf, Inf, and Inf+EFZ tri-cultures depicting multi-nucleated iMg in the infected condition and multi- and single-nucleated iMg in the Inf+EFZ condition.
(C) Representative images for analysis of local synapse density measured by density of synaptophysin+ (red) staining within a 50um radius of IBA1+ (green) iMg. Scale bar represents 50 µm.
(D) No significant difference in local synapse density across conditions compared to control. n= 4 differentiations (Uninf=4, Uninf+EFZ=4, Inf=6, Inf+EFZ=5). One-way ANOVA, Dunnett’s post hoc analysis, error bars represent SEM.
(E) No significant difference in random synapse density across conditions compared to control. One-way ANOVA, Dunnett’s post hoc analysis, error bars represent SEM.
(Ryan SK)

To confirm that differences in apparent iMg synaptophagy are not secondary to reduced numbers of synapses overall in a given culture condition, we also measured local and random (50 µm radius areas with no iMg) synapse density to ensure the uninfected microglia were not in a more synapse dense area (Figures 39A and 39C). In fact, there was no significant difference in iMg-proximal or random synapse density across all conditions (Figures 39D and 39E). This finding aligns with previous studies that demonstrate inhibition of phagocytosis by viral proteins Tat and Nef in both infected and uninfected macrophages, (Debaisieux et al., 2015; Giunta et al., 2008; Mazzolini et al., 2010), and is also consistent with ART-related impairment of phagocytosis (Giunta et al., 2011).
Figure 40: Inf+EFZ mitigates IL-1b and IL-8 production, but increases TNFα production

(A) Reverse transcriptase activity of Uninf, Inf, and Inf+EFZ tri-culture shows continual productive infection. n=3 infections, one-way ANOVA, Dunnett’s post hoc analysis, ***p<0.001; ****p<0.0001, error bars represent SEM.

(B-D) Cytokine analysis of Uninf, Uninf+EFZ, Inf, and Inf+EFZ iMg displays increase in IL-1b (B) and IL-8 (C) production in the Inf tri-culture, and Inf+EFZ had increased TNFα (D). n=3 infections, two-way ANOVA, Tukey’s post hoc analysis, *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001, error bars represent SEM.
4.3.12 EFZ reduced the production of IL-8 and IL-1b by infected iMg, but enhanced TNFa
(Ryan SK)

To validate the activation of cytokine and inflammatory pathways in infected microglia ± EFZ, we compared cytokine production between each condition in tri-culture. Starting at the time of iMg addition, we collected supernatant every 5 days until day 14 of tri-culture. RT activity confirmed that the iMg in the infected condition remain productively infected (Figure 40A). IL-8 and IL-1b were significantly increased in the infected tri-culture compared to uninfected control 5 days post iMg introduction (p<0.0001; p<0.001). IL-1b remained increased over uninfected at each timepoint; however, IL-8 decreases by day 10 (Figure 40B and 40C). The Inf+EFZ condition had consistent, significant reduced cytokine production of IL-8 from D10 through D14 (p<0.0001) and IL-1b at D5 (p<0.01) compared to Uninf. Uninf+EFZ had the lowest levels of cytokine production across all the groups (Figures 40B-40D). In addition, there was no increase in TNFa in the Inf tri-culture; however, the Inf+EFZ condition did have increased TNFa compared to all other conditions at D5 (Figure 40D). The Inf and Inf+EFZ tri-cultures also did not exhibit increases in IL-6 or IL-10, similar to our mono-culture findings (Figures 26H and 26I). These data combined with the scRNA data suggest there are distinct influences on the inflammatory response during infection alone and infection with EFZ. Inf iMg exclusively had increased expression of *IL-8* and *IL-1b* genes (Figure 31C). In addition, IL-8 and NF-κB signaling were activated only in Inf iMg compared to Uninf by scRNAseq.
Interestingly, TNFa regulates AP-1 (FOS) (Clark, Vagenas, Panesar, & Cope, 2005), a CD40 signaling pathway gene that is upregulated in Inf+EFZ iMg when compared to the Uninf (Figures 31C and 32D). These results further suggest distinct inflammatory reactions from Inf and Inf+EFZ that are mostly observed in the iMg.

4.4 Discussion

We describe a novel HiPSC tri-culture model to investigate the interdependent and individual roles of microglia, astrocytes, and neurons in the context of HIV infection. In recent years, there have been several new differentiation protocols for these cell types (Abud et al., 2017; Santos et al., 2017; Yingsha Zhang et al., 2013) and several iPSC/primary cell co-cultures that have studied various neurological disorders (Y.-T. Lin et al., 2018; Park et al., 2018). This model advances prior works in exploring gene expression changes with scRNA sequencing in the context of viral infection and relevant drug treatments to begin to unfurl a particularly difficult disease to study.

This model recapitulates several prior in vivo findings, such as increases in IL-8 and IL-1b production in microglia and EIF2 signaling in astrocytes and neurons (Akay et al., 2012; Ginsberg et al., 2018). The consistent activation of EIF2 signaling in all three cell types not only recapitulates previous findings, but also suggests microglia have dysregulated EIF2 signaling. ISR and EIF2 activation been implicated in establishing initial viral replication (Jiang et al., 2017). A recent study also found stress response genes, including the ISR-associated gene ATF4, upregulated in the brains of older HIV-positive patients
The UPR and consequently EIF2 activation has been implicated in multiple neurodegenerative disorders (Scheper & Hoozemans, 2015), these findings bolster the possibility that UPR and ISR activation play important roles in HAND development or at least the initial response to HIV in the brain.

We were also found that the gene pathways associated with IL-8, IL-1b, and TNFα production were upregulated in iMg, suggesting the microglia are the main culprit in the initial response to HIV infection; however, iMg infection seems to lead to reduced phagocytosis of synapses. This falls in line with previously reported impaired phagocytosis by HIV-infected or exposed macrophage (Debaisieux et al., 2015; Mazzolini et al., 2010). While random synapse density was variable, there was consistent reduced synaptic particles in Inf iMg, suggesting there was reliably a surplus of synapses. Although not significant, Uninf+EFZ cultures had consistently less overall synapse density, suggesting the reduced phagocytosis may be due to a global reduction in synapses. In addition, we cannot rule out the possibility that the Inf or Uninf+EFZ were more efficient in degrading the synaptic particles. Ideally, future studies using live-imaging are warranted to resolve this issue. How iMg phagocytosis would be affected or if inflammation patterns would change in longer-term cultures remain to be determined.

In addition, we discovered a starkly different immune response at the gene and functional level in Inf versus Inf+EFZ, characterized by distinct CD40/RhoGDI pathway activation and TNFα production. CD40 and RhoGDI
activation could be controlled by the increased production of TNFa, that was only found in the Inf+EFZ condition. This persistent immune activation highlights the need for further studies into the role that antiretrovirals play in propagating the chronic inflammation seen in HIV patients today (Kolson, 2017; Saylor et al., 2016). The mitigated but distinct reaction we observed with EFZ treatment is consistent with human studies showing a reduced severity of HAND with the widespread use of antiretroviral therapies (Saylor et al., 2016), but also with its persistence despite control of viral load. Further, we revealed a substantial immune response to EFZ treatment alone. This warrants further study, particularly due to the recommended prophylactic use of ART in HIV negative patients (Spinner et al., 2016).

HiPSC cultures are particularly useful for studying HIV neuropathology, since human primary neuronal cells and postmortem tissue are limited in availability and not amenable to molecular manipulation. In addition, HIV only infects human cells, rendering the interpretation of results from animal models more convoluted. Hence, mechanistic studies of the influences of HIV and ART on human neural cells are limited. This tri-culture system allows us to better study the mechanisms of early HIV infection in the brain; however, there are caveats to this system that must be considered. Each of the iPSC-derived cell types are similar to their in vivo counterparts by gene and protein expression, as well as, function, but are not exact. In addition, the iCells’ gene expression profile at the end stage of differentiation is relatively immature and more closely represents early stages of development in vivo. Our culture system allows
reductionist study of three key cell types over weeks but may be refined by inclusion of additional cell types, and further optimization to model HAND in the adult and/or chronic setting.

This tri-culture has validated several findings in the field as well as produced multiple novel findings for HIV neuropathology. However, this model is not restricted to HIV neuropathology and can also be utilized to study other neurological disorders. This highly tractable, reductive system can be genetically and pharmacologically modified at any stage. These differentiations could also be used on patient-derived cells, creating a disease-relevant, patient-specific tri-culture system. Similar cultures have been developed (Haenseler et al., 2017; Park et al., 2018), but not to this complexity, as rapid a differentiation, or with a focus on viral infections. The novelty of the tri-culture is not the individual differentiations of iMg or iAst, as there are many published differentiations. Rather, it is the first all HiPSC tri-culture that reliably recapitulated the intricate interactions among multiple cell types during HIV infection and revealed new, potential therapeutic targets. Alternatively differentiated iNrs, iMg, or iAst or additional cell types could be implemented. The tri-culture can be another, instrumental tool in understanding the workings of complex neurological disorders and in developing novel therapeutic strategies.

5. Future Directions

5.1 Conclusions

We utilized HiPSC-derived, disease relevant cell types to elucidate mechanisms, functions, and cell-cell interactions of two complex, neurological disorders, schizophrenia and HAND. We discovered mitochondria related defects in Complex I and IV of the electron transport chain and impaired ATP production in 22qSZ iNrs compared to healthy control. We also found impaired barrier properties in BBB endothelial cells derived from 22QSZ individuals that lead to increased transmigration and inflammation of immune cells. Lastly, we validated a novel tri-culture of iPSC-derived neurons, astrocytes, and microglia that largely represent the initial inflammation response to HIV infection in the brain. In addition, this system revealed distinct activation of RhoGDI and CD40 signaling in the infected microglia exposed to ART, as well as pan EIF2 signaling activation across all three cell types during infection only. These findings reveal potential new therapeutic targets that may focus on improving mitochondria health and/or anti-inflammation in schizophrenia patients and ISR inhibition and anti-inflammation in individuals with HIV infection. While there are some flaws, overall these models provide invaluable insight into diseases that other models cannot. These systems built the foundation that we now can improve upon to elucidate more mechanisms as well as study other neurocognitive and neuropsychiatric diseases.
Mitochondria deficits were discovered in iNrns from 22qSZ individuals. Barrier integrity anti-inflammatory impairments were found in iBBB from 22qSZ individuals. It is unknown if 22q(-)SZ will have the same mitochondria or barrier deficits. Increased inflammation and EIF2 signaling and decreased phagocytosis were found in the tri-culture ± HIV and EFZ. It is unknown how other ART may affect inflammation and phagocytosis or if ISRIB can rescue the phenotype.
5.2 Employ iNeurons with functioning NMDA receptors

Glutamate-mediated ecotoxicity accounts for a significant portion of the synaptodendritic damage in HAND (O'Donnell et al., 2006; Saylor et al., 2016). Glutamate works through NMDA receptors with either the heteromeric dimer NR2A/NR2B or the homomeric dimer NR2B/NR2B subunits (O'Donnell et al., 2006). The iNrs produced through the Ngn2 protocol express the NMDA receptor genes NR1 and NR2; however, the iNrs do not have functional NMDA receptors, most likely due to their overall immature state by DIV21 (Yingsha Zhang et al., 2013). Individuals with schizophrenia do not usually exhibit psychosis until their early to mid-twenties (R. Li, Ma, Wang, Yang, & Wang, 2016). Increasing the maturity and electrophysical properties of the Ngn2 iNrs will make them a more representative model, especially for mitochondria-related deficiencies. Recently, Eggan’s group showed that by combining the Ngn2 protocol with dual SMAD and WNT inhibition, roughly 35% of the iNrs will have functional NMDA receptors when co-cultured with mouse glia by DIV28 (Nehme et al., 2018). By implementing the SMAD/WNT inhibition in the iNrn differentiation, the tri-culture system can better represent the pathology seen in vivo.

Current experiments are being performed to implement the SMAD/WNT inhibition into the Ngn2 protocol during the tri-culture differentiation. The iNrs will be tested for functional NMDA receptors via NMDA-mediated toxicity, calcium-sensitive dyes, and electrophysiology. Once confirmed, we can begin to determine how microglia and astrocytes each contribute to glutamate production.
and mediated damage on the iNrrns in the context of HIV infection. Additionally, we will be able to determine how various combinations of ART affect glutamate-mediated synaptodendritic damage. Before the advent of ART, the major pathological manifestation of HAND was overt neuron loss. With ART, the major manifestation is synaptodendritic damage (Saylor et al., 2016). Inflammation is significantly reduced with ART, although still present, and it is still not known exactly what is causing the residual synaptodendritic damage. While the tri-culture is able to replicate early HIV infection into the brain, synaptodendritic damage has not been found by DIV21, when the tri-cultures are stopped for analysis. By inducing functional NMDAR by SMAD/WNT inhibition and increasing tri-culture timelines to at least DIV28, we may be able to replicate the chronic, synaptodendritic damage seen in vivo and thus study it in the reductive, manipulatable all human tri-culture. This would be an advance over other in vitro cultures that focus on acute neuronal damage in a 24 hour exposure using rodent neurons and human monocyte derived macrophages (O'Donnell et al., 2006).
Figure 42: Oxyblot analysis of HC versus 22qSZ iNrsns

(A) Oxyblot and b-actin control western for HC and ss2SZ neurons
(B) Quantitative analysis of oxyblot, normalized to b-actin. n=3 (4 HC lines, 3 22qSZ lines). ns=not significant. Error bars represent SEM.

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In addition, utilizing the mature and active iNrsns can help elucidate the downstream effects of the mitochondria-related deficits we found. With the increased activity, the iNrsns would need to use more ATP. Since there are deficits in complexes I and IV and ATP production in the 22qSZ, the increased activity may reveal functional deficits that we were not able to find previously. This could include increased reactive oxygen species production as well as, impaired electrophysiology properties, which have been both indicated in previous 22qSZ studies (Brennand et al., 2015; Devaraju et al., 2017). Our initial study only showed minor differences in electrophysiological properties that were mostly not statistically different and no significant change in oxidative damage.
(Figure 42A and 42B); however, the neurons were still relatively immature and were not exposed to any stressor. Overall, more mature, active iNrms could help uncover phenotypes that were previously not seen in the either culture system that are relevant to disease progression.

5.3 Employ more mature iAstrocytes

The astrocyte component of the tri-culture plays a number of crucial roles in maturation of neurons by increasing synaptogenesis through factors like thrombospondin-1 (Christopherson et al., 2005), developing synaptic plasticity with its role in the tripartite synapse (Araque et al., 1999), and regulating glutamate homeostasis (Santello et al., 2019), among others. It is also involved in inflammation during HIV infection, even though they are not productively infected, and other disease states (C. Akay et al., 2012; Liddelow et al., 2017). Astrocytes are extraordinarily important in assisting the tri-culture to replicate homeostatic and disease states.

The iAstrocytes used in these studies fulfill several homeostatic and disease state requirements. They produce thrombospondin-1, which is required for early synaptogenesis (Christopherson et al., 2005). The iAst gene expression profile is representative of early, fetal astrocytes, so representative of their in vivo counterparts, but not quite as mature as would match the disease states we are modeling. In addition, they propagate calcium waves in a gap-junction dependent manner. The iAst also produce a representative immune response. When exposed to IL-1b, a cytokine shown to be expressed by HIV-infected microglia by
our data and others (Walsh et al., 2014), the iAst responded with significant expression of IL-1a. The iAst also seemed to respond to another HIV relevant cytokine, IL-8, but it did not increase cytokine expression to a statistically significant level. Our findings may be due to the relatively short exposure time of eight hours. In addition, scRNAseq analysis of the tri-culture revealed that the iAst had increased EIF2 signaling activation in the infected and EFZ alone treatments. Astrocytes have increased EIF2α phosphorylation in HAND patients as well (C. Akay et al., 2012). EIF2 signaling has been implicated in cytokine production (Shrestha et al., 2012). Overall, the iAst represented their in vivo counterparts in the tri-culture in both homeostasis and inflammation relatively well, but their relative immaturity and limited immune response can be improved upon.

In order to improve the iAst component of the tri-culture, the lab is exploring two options. The first is increasing the time of the tri-culture. This approach goes in hand with the iNrn maturation. Many functions of the iAstrocytes are activity dependent and require exposure and interactions with neurons (Perego et al., 2000). In the paradigm of the study, the iAst were only exposed to iNrs and iMicroglia for two weeks. By extending the cultures, we could see increased glutamate uptake, increased synaptogenesis, and a more robust immune response. The second option being explored is to use another astrocyte differentiation protocol. There are several other published protocols that lead to iAstrocytes with GFAP, GLT-1, and GLAST expression as well as robust
inflammatory responses (Santos et al., 2017; Tchieu et al., 2019; Wang et al., 2013). Implementing iAst from one of these protocols or by extending the tri-culture time window may help maturate not just the astrocytes, but also the neurons and thus create a more representative model.

5.4 Develop More advanced co-cultures with iBBB

We showed increased migration across the iBBB and subsequent reduction in anti-inflammatory thrombomodulin in transmigrating CD14+ monocytes in 22qSZ. We and others have extensively shown increased inflammation in the brain parenchyma (Saylor et al., 2016) during HIV infection that contributes to HAND development. As peripheral inflammation may play a role in schizophrenia and in HAND, combining the iBBB system with the tri-culture can allow us to study the role of peripheral inflammation in both disorders. Studies have implemented co-cultures using transwells where the BBB cells are on the transwell and various cell types including astrocytes, pericytes, and neurons are below on the plate surface (Stone, England, & O'Sullivan, 2019). However, no one has used exclusively iPSC-derived cell types.

We could combine the two culture systems, with the iBBB in the transwell and the tri-culture on the bottom of the well, and study transmigrating immune cells, as well as, inflammatory factors that originate outside of the brain parenchyma. This will allow us to combine the iAst with the endothelial cells as astrocytes are a major component of the BBB (Abbott et al., 2010). We have already shown that CD14+ monocytes transmigrate through 22qSZ iBBB at
higher rates and have reduced thrombomodulin expression after migration. We also observed increased expression of IL-6 at perivascular astrocytes in both 22q mice and humans. However, we have not studied the direct effects that the transmigrating monocytes might have on the astrocytes, microglia or neurons in the context of 22qSZ. Utilizing the transwell system, we will study the direct effects of the invading monocytes on gene expression on the iNms, iMg, and iAst, as well as, cytokine expression and phagocytosis by the iMg and iAst. We will also be able to mix and match 22q ± SZ cell lines with healthy control to further pinpoint the role of each cell type and how the 22q microdeletion affects that role. In the context of HAND, our studies have focused on infection and inflammation y microglia, the resident immune cell of the brain. Virus initially enters the brain via transmigrating immune cells (Atluri et al., 2015; Saylor et al., 2016). We could employ the same iBBB and tri-culture paradigm but use HIV infected MDMs instead of CD14+ monocytes to study how the role of migrating, infected cells during early infection, as well as how, HIV infection may affect BBB integrity. Overall the combined system would be a powerful tool that will allow us to mechanistically study each cell type and each genotype while they are interacting with each other in a relevant multi-cell culture system.

5.5 Comparing healthy control, 22qDS without SZ, and 22qSZ lines

While 22qDS is the largest genetic risk factor for schizophrenia, only 25% of individuals with 22qDS develop schizophrenia (Chow et al., 2006). In our studies, we used 22qSZ lines, making us unable to determine if the mitochondria deficits were specific to the subset of 22qDS individuals that develop
schizophrenia or if those deficits are present in all 22qDS patients regardless of schizophrenia diagnosis. If the former is true, there may be a compensation mechanism in the 22qDS without SZ (22q(-)SZ) individuals that allow for normal mitochondria function. If the latter is true, there is likely additional mutations and/or environmental stressors that in combination lead to the development of schizophrenia. To begin to answer this question, we have obtained 22q(-)SZ lines along with additional HC and 22qSZ lines from the Pasca lab. With all three conditions, we can begin to determine if the mitochondria deficits are schizophrenia specific.

5.6 Organoids
Organoids are another advanced iPSC-based culture system that allows for more complex and representative interactions among cells by being in a 3D structure. While brain organoids develop neurons and eventually astrocytes, they will not naturally develop microglia, as they come from a different lineage. In collaboration with Kim Christian’s group, we have shown that our iMicroglia can engraft into the organoids (Figure 43). Ongoing studies will test the effects of various antiretrovirals on the health of the organoids. We also are setting to further explore the effects of HIV infected microglia with and without ART in the organoid system. Using organoids instead of the tri-culture will allow us to answer different questions, including hypotheses on neuronal patterning changes that may be impaired. In addition, we plan to use this system to screen the effects of drug abuse, which is a common comorbidity in HAND patients (Tedaldi, Minniti, & Fischer, 2015), with a focus on cannabinoids. In sum, the organoid
system will allow us to ask different but supportive questions on the role of each cell type, ART, and drugs on HIV-related neuropathy.

Figure 43: iMicroglia successfully engraft into organoids

Organoids were engrafted with iMicroglia and fixed five days later. Organoids were sectioned and stained for DAPI (grey), IBA1(green), PAX6 (red), and CTIP2 (blue). Courtesy of Jordan Scholl, Fadi Jacob, and Kim Christian.

5.7 Evaluate the effects of other frontline antiretrovirals

In our studies, we focused on the non-nucleoside reverse transcriptase inhibitor, NNRTI, Efavirenz. Since NNRTIs prevent new infection, but do not prevent previously infected cells from producing virions, we were able to study HIV infected and uninfected, but activated microglia in the same culture by introducing EFZ six days post infection. However, there are fusion inhibitors, entry inhibitors, nucleoside reverse transcriptase inhibitors, integrase strand transfer inhibitors, and protease inhibitors as well. Each of these drug classes
target a specific part of the HIV replication cycle and can have their own side
effects. In fact, protease inhibitors activate the UPR stress pathways in several
cell types including MDMs and impair oligodendrocyte maturation (Jensen, Roth,
Grinspan, & Jordan-Sciutto, 2019). How these different drugs by themselves or in
combination negatively affect microglia, astrocytes, and neurons has not been
fully determined. We could use the tri-culture system with the same output
measures to determine the effects of each drug class by itself and in combination
on the gene expression of each cell type, the overall inflammation response and
effect on phagocytosis, as we found that EFZ treatment alone was enough to
impair synaptophagocytosis. This can be done in conjunction with the proposed
BBB / tri-culture to build a more physiologically relevant model that accounts for
antiretroviral penetration through the BBB. These studies are not only relevant for
patients with HIV, but also for individuals that use Pre-exposure prophylaxis,
PrEP. Many individuals who are at high risk to HIV exposure proactively take the
NRTIs tenofovir and emtricitabine (Saag et al., 2018). It is not well understood
how chronic use of PrEP for years affects CNS homeostasis and development,
especially in adolescents whose brains are still developing. The tri-culture can
help ascertain the effects of chronic use of PrEP unlike other models that focus
on acute changes. We have shown that the tri-culture can effectively model the
inflammatory response of EFZ during chronic use with and without HIV infection.
We can therefore study other drugs and combinations of drugs in those same
paradigms.
5.8 Evaluate the role of PERK haplotype in HAND progression

The tri-culture system can also be a powerful tool for studying genetic variants associated with the disorder and how those variants affect different cell types. EIF2 signaling was activated in all three cell types during infection. EIF2 phosphorylation is regulated by the Integrated stress response, ISR. The ISR responds to several cellular stressors including, amino acid deprivation, endoplasmic reticulum (ER) stress, viral infection, and heme deprivation, and can be activated through one of four kinases, GCN2, HRI, PKR, and PERK (Bond et al., 2020). All kinases lead to phosphorylation of EIF2α which subsequently leads to global translational repression. Our lab has previously shown that EIF2 activation is increased in HAND (C. Akay et al., 2012). The PERK arm of the ISR is activated through ER stress is part of the unfolded protein response (Janssens et al., 2014). The PERK gene, *EIF2AK3*, has four haplotypes, A, B, D, and E. Almost 70% of the population has haplotype A and roughly 30% of the population has haplotype B. Haplotype D and E account for less than one percent. Haplotype B has increased PERK kinase activity and been associated with increased risk for progressive supranuclear palsy and Alzheimer’s disease (Stutzbach et al., 2013). Haplotype B has 3 nonsynonymous SNPs in the PERK open reading frame when compared with haplotype A, rs867529, rs13045, and rs1805165 (Figure 44B and 44C). rs867529 and rs13045 are located in the coding region for the ER stress sensing domain in the ER lumen and rs1805165 is located in the coding region for a functionally unknown position in between the split kinase domain in the cytoplasm portion of the transmembrane protein.
(Figure 44A and 44C). (J. Liu et al., 2012; Ma, Vattem, & Wek, 2002). The HiPSC system will allow us to study the potential role of haplotype B in a number of disease contexts by generation of isogenic lines using through gene editing.

We can use HiPSC lines that are from haplotype A and B patients as well as use CRISPR-Cas9 to change haplotype A to haplotype B and vice versa, creating isogenic controls. We are currently sequentially editing the three SNPs on a haplotype A cell line. The first SNP that we are editing is rs13045. The sequential editing will also allow us to partially determine the importance of each SNP in PERK kinase activity. More importantly, because we will be editing at the iPSC stage, we can choose the haplotype of each of the three cell types, allowing us to clearly define the role of haplotype B in each cell type during HIV infection. Overall this system allows us to rapidly and selectively genetically manipulate each cell type to further understand how genetic variants can affect disease progression.
Figure 44: PERK structure and SNPs

(A) PERK has an ER lumen and cytoplasm domain. Within the cytoplasm domain, there is a kinase domain with an insert region dividing the kinase domain in two.

(B) The PERK gene consists of 17 exons. 3 SNPs of interest, rs867529, rs13045, and rs1805165 are located in exons 2, 3, and 13 respectively.

(C) Locations of each SNP with the amino acid number and the respective amino acid for haplotypes A and B at those locations.

(D) Base pair change from haplotype A to B at each SNP.

Adapted from (J. Liu et al., 2012; Ma et al., 2002; Yuan et al., 2018).

5.9 Treat activated pathways and develop the tri-culture as a drug screening platform

EIF2 signaling was activated in all three cell types in the tri-culture during infection and EFZ treatment alone. It was still activated in the iAst and iNrrs during Inf+EFZ. A commercially available inhibitor of the ISR, ISRIB, allows for global translation despite EIF2α phosphorylation. We can use this drug to determine EIF2 signaling activation is responsible for the activation of the other neuroinflammatory pathways, such as, NF-κB signaling and NRF2-mediated
oxidative stress response in the Inf only and EFZ only iMg, and RhoGDI and CD40 in the Inf+EFZ iMg. It will also allow us to determine if the cytokine response is downstream of the ISR pathway. Additionally, we can specifically block the PERK pathway of the ISR with perk inhibitors to begin to determine which kinase or kinases of the ISR are specifically activated. These experiments could directly determine the upstream causes of the inflammation response we observed in the tri-cultures.

In conjunction with specific targeting of the EIF2 signaling pathway, the tri-culture could be used as a drug screening platform on a broader scale. Since the tri-culture can use any human cell line, any genetic disease, including schizophrenia, autism, and Huntington’s, can be studied by using patient lines. In addition, other virus-related pathologies, including Zika can also be studied. Potential therapeutics can be tested on the tri-cultures in human, disease relevant cell types that are able to interact with each other. Which is a large advantage of the standard HEK, CHO, or HeLa cell lines that are often used for drug screening. Overall, the tri-culture system provides a novel model system not just for mechanistic work, but also as a powerful drug screen tool.

iPSC-based model systems are a powerful new tool that allow researchers to study the mechanisms of complex disorders in reductive, tractable systems that include the genetic variation that so often complicates said diseases. Our work has revealed mitochondria-related defects in HiPSC-derived neurons and blood brain barrier impairment from patients with 22q11.2 with schizophrenia. In
addition, we have developed a tri-culture that has allowed us to study the cell-cell interactions in the context of early HIV infection and antiretroviral treatment in the brain, revealing therapeutically targetable pathways. By utilizing these models and integrating other iPSC-based platforms, such as organoids, we can better define the etiology of not just neurological disorders, but also diseases of any system.
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