Characterizing the Role of Histone Deacetylase 2 (HDAC2) in Mitochondria Function and Alzheimer's Disease

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Abstract

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Histone deacetylase 2 (HDAC2) is a major HDAC protein in the adult brain and has been shown to regulate many neuronal genes. The aberrant expression of HDAC2 and subsequent dysregulation of neuronal gene expression is implicated in neurodegeneration and brain aging. Human induced pluripotent stem cell-derived neurons (hiPSC) are widely used models for studying neurodegenerative disease mechanisms, but the role of HDAC2 in hiPSC differentiation and maturation has not been explored. In this study, we show that levels of HDAC2 progressively decrease as hiPSCs are differentiated towards neurons. This suppression of HDAC2 inversely corresponds to an increase in neuron-specific isoforms of Endophilin-B1, a multifunctional protein involved in mitochondrial dynamics. Expression of neuron-specific isoforms of Endophilin-B1 is accompanied by concomitant expression of a neuron-specific alternative splicing factor, SRRM4. Manipulation of HDAC2 and Endophilin-B1 using lentiviral

approaches shows that the knock-down of HDAC2 or the overexpression of a neuron-specific Endophilin-B1 isoform promotes mitochondrial elongation and protects against cytotoxic stress in hiPSC-Ns, while HDAC2 knock-down specifically influences genes regulating mitochondrial dynamics and synaptogenesis. Furthermore, HDAC2 knock-down promotes enhanced mitochondrial respiration and reduces levels of neurotoxic amyloid beta peptides. Collectively, our study demonstrates a role for HDAC2 in hiPSC-neuronal differentiation, highlights neuron-specific isoforms of Endophilin-B1 as a marker of differentiating hiPSC-Ns and demonstrates that HDAC2 regulates key neuronal and mitochondrial pathways in hiPSC-Ns.

TABLE OF CONTENTS

LIST OF FIGURES

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To my late father – Mr. Akwasi Yeboah. Thank you for your sacrifice to get me here. The lessons of hard work, respect and excellence remain the foundation on which I build my life and for that, I say thank you. Continue to Rest in Peace Dad!

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Chapter 1. INTRODUCTION

1.1 ALZHEIMER'S DISEASE

1.1.1 *Alzheimer's Disease Pathophysiology, Current Treatment and Trials*

Alzheimer's Disease (AD) is a progressive neurodegenerative disease characterized by the presence of amyloid beta (aβ) plaques and neurofibrillary tangles (tau). Aβ peptides are derived from the cleavage of the amyloid precursor protein (APP) by β-secretases and γ-secretases (known as the amyloidogenic pathway) or by α-secretase to soluble APPα (known as the non-amyloidogenic pathway) (Vassar et al. 1999; Sisodia 1992; Haass et al. 1993). Tau, on the other hand, is a microtubule-associated protein that helps to stabilize microtubule structure and regulate the movement of organelles and molecules along the axons of neurons (Eftekharzadeh et al. 2018; V. M. Lee, et al. 2001). Post-translational modification of tau results in a hyperphosphorylated state (pTau) which accumulates in the somatodendritic compartment of neurons (Iqbal and Grundke-Iqbal 2006; Dujardin et al. 2020). 3R and 4R are the tau species observed histologically in AD. The interaction of Aβ and tau with the neuronal synapse and their subsequent effect fits the classification of AD as a synaptic dysfunction disorder. Although Aβ is produced by most cells, its production is particularly high in synapses during synaptic activity and it also tends to aggregate (Cirrito et al. 2005). Moreover, tau is primarily present within the cytoplasm of neuronal axons but has also been shown to be present in both presynaptic and postsynaptic compartments (Pooler, Noble, and Hanger 2014). Other observed phenotypes in AD include epigenetic alterations, enlarged endosomes, activated glia, and impaired protein homeostasis (Cataldo et al. 2000; Menzies et al. 2017; Hong et al. 2016; Kwart et al. 2019).

AD can broadly be grouped into sporadic AD and early-onset familial AD (Bateman et al. 2011; Bali et al. 2012). Sporadic AD occurs with no clear genetic cause and typically develops after the age of 65. It is the most common form of AD, accounting for approximately 95% of cases. The early-onset familial AD is a rare form of AD caused by mutations in certain genes, such as APP, presenilin 1 (PSEN1), and presenilin 2 (PSEN2) (S. J. van der Lee et al. 2018). This usually develops before the age of 65 and accounts for less than 5% of all AD cases. Using whole-exome and whole-genome sequencing, a cohort study of 1779 AD cases (852 early and 927 late onset) and 1273 controls among other studies have further confirmed the contributions of these genetic variants to AD risk (Bellenguez et al. 2017). The percentage contrast between the total cases of familial and sporadic AD cement aging as the main risk factor for AD. Recently sex differences have also been reported in AD with women having a higher disease prevalence in most studies (Mielke, Vemuri, and Rocca 2014; Ferretti et al. 2018).

Biomarkers from the blood, cerebrospinal fluid, and imaging along with cognitive tests are used to diagnose and identify the stage of AD progression in a patient (Jack et al. 2018). These tests also help separate AD from other tauopathies such as frontotemporal dementia(FTD), Pick's disease, Progressive Supranuclear Palsy (PSP), and Chronic Traumatic Encephalopathy (CTE). The severity of cognitive impairment in patients with AD varies and the earliest signs could be a decline in mental and cognitive abilities. This is further facilitated by lifestyle (exercise, diet, sleep cycle) or underlying diseases such as diabetes or hypertension. For example, it has been shown that Aβ levels are modulated by the sleep–wake cycle. Thus there is higher Aβ release during wakefulness and higher clearance during sleep (Sabia et al. 2021; Boespflug and Iliff 2018).

The prevalence of all causes of dementia including Alzheimer's is expected to increase from 50 million people in 2010 to 113 million by 2050 worldwide (Brodaty et al. 2011). Given the advances in healthcare and medical interventions, the average lifespan has been extended, which points to more individuals living to the age of 80 and over – a major blessing as well as a concern for developing AD. Yet, pharmacological interventions for AD have trailed behind other diseases such as cancers. Memantine, donepezil, rivastigmine, and galantamine are the main treatments still used in the clinic despite their modest results (Fink et al. 2018). Most drugs targeting amyloid beta or tau have failed in clinical trials with the most recent being aducanumab a monoclonal antibody that targets Aβ protofibrils from Biogen and Eisai Pharmaceuticals (Panza et al. 2019; Espay 2021; VandeVrede, Boxer, and Polydoro 2020). Biogen recently obtained accelerated FDA approval for lecanemab, an Aβ-targeting therapy after slowing cognitive decline by 27% decline in a phase III study despite very serious side effects (FDA Press Release 2023; van Dyck et al. 2023). This approval was received with mixed feelings within the scientific community and points to the need to further understand AD pathology and progression to develop an effective therapy.

1.1.2 *Mitochondria Dysfunction in AD*

In 2004, Shaharyar Khan's group proposed the mitochondrial cascade hypothesis of AD in an attempt to explain various phenomena that could not be explained solely by the amyloid cascade hypothesis (Swerdlow and Khan 2004). Cellular function and integrity decline with age and cells respond by activating various compensatory mechanisms (López-Otín et al. 2013). However, these mechanisms are not enough to sustain cellular function long-term. The mitochondrial cascade hypothesis postulates that the age at which AD ensues is determined by a person's genetically determined mitochondrial starting point, in addition to their genetically and

environmentally influenced rate of mitochondrial decline (Trifunovic et al. 2004). Hence the phenotypes of AD such as tau hyperphosphorylation, Aβ deposition, and synaptic loss are a response to the mitochondrial compensation or its failure. Also, given that the maternal mitochondrial DNA is passed onto offspring and correlating evidence from epidemiological studies that showed that AD fathers contributed less to the risk of AD, many believed the mitochondrial hypothesis as a valid approach relative to the amyloid beta cascade hypothesis (Duara et al. 1993; Edland et al. 1996). Whether the amyloid or mitochondria cascade remains the ideal theory for AD can only be proven by clinical therapy.

It is noteworthy that the initial stage of AD is marked by reduced mitochondria biogenesis and mitochondria that are functionally and morphologically impaired, making them susceptible to increased ROS production and decreased ATP production (Gomes and Santos 2013; Du et al. 2008; Shin et al. 2011; Hauptmann et al. 2009). This has been attributed to the apparent increase in amyloid beta levels and its subsequent effects. For example, amyloid beta binds to the mitochondrial matrix protein to form Amyloid-β-binding alcohol dehydrogenase (ABAD), which in turn induces the free radical formation and subsequent neuronal apoptosis (Yan and Stern 2005). Also, APP induces mitochondrial fragmentation by altering the levels of mitochondrial fusion and fission proteins and also disrupts axonal transport leading to defective mitochondrial accumulation in the neuronal axons (Goiran et al. 2018; Mohd Waseem Akhtar et al. 2016). The rapid synaptic activity in the brain is facilitated by the presence of high cellular energy and the reduced expression of mitochondria complexes and their activity leading to low ATP production in AD models further iterates the presence of mitochondria dysfunction (Cai and Tammineni 2017; Beck et al. 2016). AD pathology is also further exacerbated when Endophilin B1, a mitochondrial protein, is lost (D. B. Wang et al. 2015). In maintaining cellular

homeostasis, mitophagy is compromised in AD tissues with altered levels of Pink1 and Parkin (Twig et al. 2008). This further underscores the point that mitochondria are not isolated organelles and their defects may interplay with other downstream pathways that subsequently drive Alzheimer's disease pathology.

1.2 EPIGENETICS

1.2.1 *The Landscape of Epigenetics*

The term epigenotype and subsequently epigenetics was coined by Conrad Waddington in 1942 where he argued that there is a set of complex processes in between development that link a genotype to a phenotype (Waddington 1942). Unlike the genetic mutations that affect the underlying DNA sequence of various genes, epigenetic alterations are changes in gene expression or function that can be inherited; but do not make any changes to the primary DNA sequence. These post-translational modifications (PTM) include phosphorylation, acetylation and methylation. (Egger et al. 2004). The resulting effect of the crosstalk between these three categories coupled with both feed-forward and negative regulatory mechanisms gives the final epigenetic output. Therefore methylation may have an effect on histone modification and vice versa. Epigenetic changes can be affected by several factors such as environmental exposures, age, lifestyle, and diet. Additionally, multiple studies have pointed to their roles in embryonic development, aging, and disease (Haberland, Montgomery, and Olson 2009; Goelz et al. 1985; López-Otín et al. 2013). For example, patients with colorectal cancers had more DNA methylation in their healthy tissues as compared to their diseased tissues(Feinberg and Vogelstein 1983). Also, methylation turns off about 40% of genes that account for inherited forms of cancer patients through a phenomenon known as epigenetic silencing. From a

developmental standpoint, the effect of epigenetic silencing contributes to the differences between twins and the X-chromosome inactivation in female mammals (Egger et al. 2004).

1.2.2 *Acetylation: KAT and HDACs*

Acetylation diminishes the electrostatic affinity between neighboring histones and the DNA and, consequently, can promote a more open chromatin structure that allows for gene transcription. On the reverse, deacetylation is marked by a reduction in histone acetylation at the gene promoters, which subsequently results in chromatin condensation and gene silencing. The role of histone acetyltransferases (HATs) or lysine acetyltransferases (KATs) and histone deacetylases (HDACs) are responsible for the transfer and removal of acetyl groups. Acetylation is a conserved post translational modification that adds acetyl groups to proteins, with the epsilon amino groups on the lysine of the proteins as the main target. This process is facilitated by enzymes formerly called histone acetyltransferase (HATs) but now generally called lysine acetyltransferases (KATS). Initially, the activity of KATS was deemed irreversible, however, the work of Allfrey et al in 1964 showed their reversible effects on histones (Allfrey, Faulkner, and Mirsky 1964). Since then researchers have placed emphasis on discovering and characterizing various enzymes believed to have similar and parallel catalytic activity like the reported KATs. As of 2019, Bilal et al reported 37 discovered KATS with function ranging from DNA recognition and repair, protein stability, chromatin organization, and enzyme structure (Choudhary et al. 2014; Roth, Denu, and Allis 2001; Marmorstein and Zhou 2014; K. K. Lee and Workman 2007). The major studied acetyltransferases are categorized into three families: the p300/CBP family, the MYST family, and the GCN5/PCAF family. With advanced biochemical assays and experimental approaches, the discovery of more KATS will be rapid as well as novel functions they may play in human physiology and disease. Yet aside from the canonical roles

KATs play, KATs have also been shown to acetylate non-histone proteins amongst other functions. Acat1 as an example catalyzes the final stage in the degradation of ketone bodies but has also been shown via in vitro experiments to catalyze acetylation of purified PDH1 and PDP1 (Yang et al. 2004; L. Chen et al. 2019; Fan et al. 2014).

HDACs (Histone deacetylases) are a family of enzymes that remove acetyl groups from histone proteins, leading to a closed chromatin conformation and gene silencing. The non-specific inhibition of HDACs by classical inhibitors has hindered the ability to study individual HDAC function. As an alternative, in vivo, work using mutant mice models coupled with in vitro biochemical assays have helped delineate the specific functions of the HDACs in physiological conditions and in diseases when perturbed. It is therefore noteworthy that the reported studies in literature may seem contradictory as results from pharmacological inhibitors may differ from genetic manipulation of HDACs. This is likely because the transient and incomplete nature of pharmacological inhibition may allow for other compensatory mechanisms whereas the genetic loss of HDACs is so robust that total phenotype ablation is seen.

There are a total of 18 HDACs, which are subdivided into classes I–IV based on their homology to their respective yeast orthologues. Class I, II, and IV which consist of 11 HDACs function using a zinc-dependent catalytic mechanism and are commonly known as ‘classical’ HDACs (K. K. Lee and Workman 2007; Haberland, Montgomery, and Olson 2009). Taunton et al were the first to characterize a 55kD protein as the first set of mammalian HDACs which was named HDAC1, a member of the class I HDAC family (Taunton, Hassig, and Schreiber 1996). Class I HDACs (HDAC1, HDAC2, HDAC3, and HDAC8) share a similar sequence homology to the Rpd3 protein in yeast and are generally believed to be found in the nucleus and ubiquitously expressed throughout the body. However, more precise studies have also pointed to specific

locations and functions for some of the class I HDACs. Using HDAC2-deficient mice, Guan et al showed that HDAC2 is the most abundant HDAC in the brain and contributes to various processes involved in synaptic plasticity and memory formation (Guan et al. 2009). A 2018 paper by Donald Menick's group also showed for the first time that HDAC1 can localize to the mitochondria and plays a key role in cardiac reperfusion injury (Herr et al. 2018).

Class II HDACs consist of HDAC4, HDAC5, HDAC6, HDAC7, HDAC9, and HDAC10. Their shared homolog is to the Hda1 protein in yeast (Grozinger, Hassig, and Schreiber 1999). There is a broad similarity range (20-80%) between class II HDACs which are sometimes subclassified into class IIa and class IIb HDACs (Seto and Yoshida 2014; Haberland, Montgomery, and Olson 2009). Unlike class I HDACs, class II HDACs show a more restricted expression pattern in specific tissues in the body yet they all point to a cytoplasmic location. Worth noting is the fact that HDAC6 and HDAC10 have two catalytic domains not found in other HDACs (Zhang Yu et al. 2008; Kao et al. 2002). HDAC11 is the only member of the class IV HDACs and biochemical assays point to its localization in the brain, heart, and skeletal muscles (Gao et al. 2002; Liu et al. 2008). HDAC11 serves as the main deacetylase for the cdt1 gene, a crucial gene in DNA replication during the cell cycle (Glozak and Seto 2009). The Class III HDACs are normally called sirtuins. These are a seven-member family of highly conserved NAD(+)-dependent enzymes with homolog similarity to the sir2 protein in yeast. SIRT1, SIRT6, and SIRT7 are mainly localized to the nucleus, SIRT2 is mainly in the cytoplasm, and the mitochondrial matrix houses SIRT3, SIRT4, and SIRT5 (Dryden et al. 2003; Serrano et al. 2013).

1.2.3 *General functions of HDACs and potential therapeutic targets of HDAC*

Class I HDACs: In neurons, HDAC1 has been shown to play a role in neuronal progenitor cell formation and differentiation as well as elevated levels in disease models of various

polyglutamine diseases such as Huntington's Disease (Ye et al. 2009; Kuhn et al. 2007). Also, deacetylation of p65 by HDAC1 can lead to the repression of NF-κB and STAT3 target genes involved in inflammation (L.-F. Chen and Greene 2004; Gonneaud et al. 2014). Li-Huei Tsai's group also reported lower levels of HDAC1 in aging adults and Alzheimer's patients and demonstrated that activating HDAC1 was beneficial for restoring DNA damage and cognitive decline (Pao et al. 2020). Deletion of HDAC3 protects mice from diet-induced obesity and plays a crucial role in lipid metabolism (Dávalos-Salas et al. 2019). In Alzheimer's disease, a selective HDAC3 inhibitor, RGFP-966, relieved most of the disease phenotypes in HEK-293 cells overexpressing APP with the double Swedish mutation (Janczura et al. 2018). The same inhibitor induces apoptosis and disrupts tumor-associated protein expression in acute myeloid leukemia (Beyer et al. 2019). HDAC3 is also involved in colorectal cancer progression by regulating the miR-296-3p/TGIF1/TGFβ pathway (Li et al. 2020). HDAC8 has been shown to play a role in EMT, a process by which epithelial cells acquire mesenchymal characteristics and promote cancer metastasis. HDAC8 inhibition can prevent EMT and inhibit cancer cell migration and invasion (Pantelaiou-Prokaki et al. 2022).

Class II HDACs: There is a higher expression of HDAC5 in breast cancer among young females which correlates with lower survival rates. An HDAC2 inhibitor (LMK-235) was able to curb the poor prognosis and stop tumor growth (Oltra et al. 2020). On the contrary, HDAC5 expression is greatly impaired in acute myeloid leukemia and colon cancer and has been linked with poor clinical outcomes in lung cancer (Bradbury et al. 2005; Ozdağ et al. 2006). HDAC4 plays a central role in the formation of the skeleton. It is expressed in prehypertrophic chondrocytes and is essential for regulating chondrocyte hypertrophy and bone formation. Mice with a global deletion of HDAC4 die due to the conversion of cartilaginous skeletal elements to ossified bone

(Arnold et al. 2007; Vega et al. 2004). HDAC10, a class IIb HDAC has been studied for its role in lysosomal function and DNA mismatch repair function (Koeneke, Witt, and Oehme 2015).

Class III HDACs: SIRT1 is an interesting deacetylase with both beneficial and harmful reports in literature so far. These variations could be tissue-specific and cell-state-dependent. In cancers, SIRT promotes tumorigenesis by deacetylating and repressing the tumor suppressor gene p53 whereas SIRT1 protects against neurodegeneration in the p25 AD transgenic mice (D. Kim et al. 2007; Chalkiadaki and Guarente 2015). Chemical activators of SIRT1 such as SRT1460, SRT1720, and resveratrol reduce cancer incidence, extends lifespan, and show general phenotypes similar to caloric restriction (Luo et al. 2001; Milne et al. 2007). These anti-aging properties make SIRT1 a potential target for treating age-related and metabolic diseases (Houtkooper, Pirinen, and Auwerx 2012). By deacetylating α-tubulin in microtubules, SIRT2 can prevent cardiomyocyte hypertrophy and contractile impairment. SIRT3 controls the contractile function of cardiac muscles and its inhibition leads to impaired cardiac function due to an increase in fatty acid oxidation and subsequent formation of reactive oxygen species (Palomer et al. 2021). Cellular homeostasis by autophagy is also influenced by various SIRTs such as SIRT1,-2, -3, and -6 with resulting diseases such as diabetic nephropathy and various neurodegenerative diseases (I. H. Lee 2019).

Class IV HDACs: HDAC11 inhibition has been shown to be a potential approach to overcome kinase inhibitors resistance in hepatocellular carcinoma and in non-small cell lung cancer by repressing Sox2 activity (Bi et al. 2021).

Though HDACs differ in their localization, expression, and structure, some redundancies and compensatory effects have been reported in their function. For example, overexpression of HDAC8 in HeLa cervical cancer cells leads to functional redundancy with HDAC6, resulting in

deacetylation of ac-Lys40 of alpha-tubulin. This process promotes the proliferation and progression of cervical cancer (Vanaja, Ramulu, and Kalle 2018). Several studies have also reported an increase in the expression of HDAC2 when HDAC1 is inactivated, while HDAC1 is upregulated when HDAC2 is deleted (Yamaguchi et al. 2010; Haberland et al. 2010; Zimmermann et al. 2007). Taken together, histone deacetylases may have more functional abilities than already discovered and further research will delineate their unique differences while exploring the concomitant effect of one on another.

1.2.4 *HDAC2 in Focus*

HDAC2 has been studied extensively due to its ability to undergo many post-translational modifications, such as phosphorylation, acetylation, ubiquitination, and sumoylation (Ashktorab et al. 2009; Krämer et al. 2003; Sun, Chen, and Davie 2007; Tsai and Seto 2002; Alsawalha et al. 2019). It is a member of the class I HDAC family, which is primarily located in the nucleus and shares a common catalytic mechanism with the other class I HDACs. HDAC2 also comprises a conserved deacetylase domain with short amino- and carboxy-terminal extensions, which are key for localization and maintaining their stability and function. Among the class I HDACs, HDAC1 in particular has been shown to be redundant to HDAC2 in certain cellular contexts while working independently in other areas (Gonneaud et al. 2019; Montgomery et al. 2009; Brunmeir, Lagger, and Seiser 2009).

HDAC2's expression across various tissues in the body drives its implication in several diseases. In HDAC2 knockout mice, liver regeneration is severely impaired – a phenomenon attributed to the reduction in the expression levels of Ki67, a mitotic marker (Xia et al. 2013). Consistent with this report is another study that observed low expression of CDK2, CDK4, and CD1, key genes in hepatocyte replication and liver mass reconstruction (Y. Wang et al. 2013). Knocking out

HDAC2 also results in the induction of senescence in MCF7 cells by increasing the binding activity and interaction of p53-DNA. Interestingly these findings were more pronounced in the female mice compared to the male mice pointing to a potential gender-dependent effect of HDAC2 expression. In microbiology, HDAC2 has been shown to exhibit anti-influenza A virus (IAV) properties. IAV counteracts HDAC2's antiviral effects by disrupting its polypeptide level through proteasomal degradation. In A549 cells, IAV reduced HDAC2 polypeptide levels in by as much as 47%. However, this was reversed to nearly 100% when treated with MG132, a proteasome inhibitor (Nagesh et al. 2017). The deletion of HDAC2 in intestinal epithelial cells (IECs) alters intestinal homeostasis through the differentiation of secretory cells that play a role in providing physical and chemical protection barriers leading to an increase in the number of intermediate secretory cell precursors. Transcriptomic and proteomic studies of IECs have also uncovered Hdac2-dependent cellular processes, such as steroid receptor pathways and lipid metabolism (Gonneaud et al. 2019).

During bioenergetic maturation in murine cardiogenesis, the absence of Hdac2 results in the initiation of cryptic transcription in metabolic loci like CS and Ndufb9. This is a result of the buildup of H3K36 trimethylation and H3K23/H3K14/H4K16 acetylation, which corresponds to an inappropriate opening of chromatin at the transcription start site associates (TSSAs) of CS and Ndufb9 (Milstone et al. 2020). Hdac2 also forms a complex with Gsk3βas a regulatory pathway and mediates the response of cardiac to hypertrophic conditions. In most cancers, Hdac2 is part of the mechanism driving cell proliferation. HDAC inhibitors are able to arrest the subsequent proliferation of cancerous cells and inhibit their growth. In colorectal cancer, a miRNA (miR-500a-5p) was used to inhibit tumor progression and Hdac2's mechanistic properties were

observed It was reported that miR-500a-5p directly targets HDAC2 and forms part of the p300/YY1/HDAC2 axis.

In the central nervous system (CNS), genetic deletion of HDAC1 or HDAC2 in mice does not exhibit any noticeable changes in the physical or developmental traits, and the mice lived as long and bred as often as mice in the control group. However, the deletion of both HDAC1 and HDAC2 caused significant neuronal loss and resulted in the death of the animals within seven days. Neuronal differentiation from precursor cells was also blocked in the dual HDAC1/HDAC2 mice. Noticeably, this effect was specific to neurons as astrocyte differentiation in HDAC1/HDAC2 knockout mice was similar to that of the control group (Montgomery et al. 2009). This confirms previously reported work about the expression patterns of HDACs 1-11 in the rat brain. HDAC2 was absent in glia cells but present found in neurons throughout the cortex and in the neuronal layers of the hippocampus with a subset expression in oligodendrocytes – suggesting HDAC2's role in myelination and in diseases such as multiple sclerosis (Broide et al. 2007; MacDonald and Roskams 2008). In oligodendrocyte formation, HDAC2 has been shown to compete with β-catenin, a member of the Wnt-signaling pathway for TCF7L2 interaction to regulate downstream genes involved in oligodendrocyte differentiation (Ye et al. 2009).

HDAC2 has also been identified as the dominant HDAC in aging and Alzheimer's disease. There is a strong correlation between severe amyloid pathology and significant dysregulation of histone acetylation in the forebrain of the APPPS1-21 AD mice model (Govindarajan et al. 2013; Francis et al. 2009). In post-mortem analysis, AD patients showed higher HDAC2 expression levels in the hippocampal CA1 neurons compared to healthy individuals, while the levels of other class I HDAC members remained unchanged (Gräff et al. 2012; Bie et al. 2014). Another

study using the 5XFAD mouse model of AD observed elevated neuronal expression of HDAC2 in the cortex and hippocampus. Further evidence supporting the involvement of HDAC2 in memory deficits in the context of neurodegenerative diseases like AD was provided by a study using the CK-p25 mice model of AD. The study showed that after the induction of p25, HDAC2 levels were increased in excitatory neurons, which was then followed by an upregulation of HDAC2 binding to the promoters of genes associated with memory and synaptic function such as BDNF, Egr1, Fos, Camk2a, Creb1, Nrnx3, GluR1/R2, and NR2A/2B. Eventually, this results in synaptic gene repression and memory impairment (Ballas et al. 2005).

CoREST, a scaffolding protein, and SP3, a transcription factor have been shown as repressor partners of HDAC2 for synaptic gene repression (Guan et al. 2009; Yamakawa et al. 2017). When the histone acetylation in these promoter regions was restored by inhibiting HDAC2 activity pharmacologically or through genetic means, the level of the synaptic associated genes were increased (Guan et al. 2009). Even at extremely late stages of disease pathology, sodium butyrate was able to improve associative learning in these mice. And also, the recovery of memory function correlated with elevated hippocampal histone acetylation and increased expression of genes implicated in associative learning (Govindarajan et al. 2011). The converse of HDAC2, Tip 60 HAT has also exhibits a highly regulated cellular process, and hence disruption of its homeostasis triggers various cellular cascades such as the epigenetic repression of synaptic genes even before amyloid beta plaques form (Panikker et al. 2018).

To further understand HDAC2's contribution to neuronal development in the context of synapse formation and development, hippocampal cultures from young C57BL/6 mice were treated with Trichostatin A (TSA), an HDAC inhibitor and observed 18-24hrs post-treatment. Additionally, mice with conditional alleles to HDAC1 and HDAC2 were studied at various points of

development. It was reported that a decrease in the activities of both HDAC1 and HDAC2 during early synaptic development causes robust facilitation of excitatory synapse maturation and a modest increase in synapse numbers. However, a decrease in HDAC2 levels alone in mature neurons was sufficient to attenuate basal excitatory neurotransmission without a significant change in the numbers of detectable nerve terminals (Mohd W. Akhtar et al. 2009). This report further showed a potential developmental switch between HDAC2 and other HDACs. Together, these observations support the potential use of HDAC2 inhibitors as powerful therapeutics to treat memory impairment associated with synaptic dysfunction in AD and neurodegenerative diseases.

1.3 Human Induced Pluripotent Stem Cell Models of Disease

1.3.1 *Generating hiPSC Models*

Using rodent models for research and drug development has been extremely helpful and led to great discoveries although this approach comes with challenges. The obvious challenge is the fact that mice and rats are simply not humans, and therefore their physiological and genetic makeup may not be representative of what is observed in humans. Therefore, while a drug may appear safe and effective in animal studies, it may not work the same way in humans, which can lead to costly clinical trial failures due to unexpected side effects or toxicity in humans (Van Norman 2019). Rodents also have a much shorter lifespan than humans, and hence the progression of diseases may be much faster than what is seen in humans. This makes it difficult to accurately model chronic diseases like diabetes or Alzheimer's, which take years to develop in humans but only months to develop in animals. Finally, the ethical concerns of using rodents for experiments while well-meaning could be a hindrance to various modifications that are needed to study disease pathology to the fullest (Joffe et al. 2016). In a study using single cell RNA-seq,

researchers from the Allen Institute found that despite the overall preservation of some neuronal markers, significant variances between equivalent cell types in humans and mice were observed (Hodge et al. 2019). This encompassed notable changes in proportions, layering patterns, gene expression, and physical characteristics. These unique attributes specific to each species highlight the crucial need to directly investigate the human brain as other studies have also highlighted the remarkedly difference between human and rodent brains (Beauchamp et al. 2022).

Human pluripotent stem cells (hPSCs), including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), on the other hand, is an alternative to research and drug development because of their relevance to human pathobiology, their availability and potential for translation studies. Furthermore, the recent FDA Modernization Act 2.0 that bypasses the requirement for animal testing for drug development further cements the usefulness of models such as of pluripotent stem cells (Han 2023). Human pluripotent stem cells have the ability to differentiate into almost any type of cell and mimic essential characteristics of human cells. Since their discovery in 2007, various improvements in the differentiation protocols continue to shorten the time and improve the yield of the desired cell types from the pluripotent state (Takahashi et al. 2007). For example, in 2009, Chambers et al reported the use of Noggin and SB431542, two inhibitors of the SMAD signaling pathway as a means to induce rapid neural conversion of hPSCs into various brain cell types based on culture media. Additionally, 3D culture systems from stem cells (organoids) are been used as a model to re-create the architecture and physiology of human organs in remarkable detail (J. Kim, Koo, and Knoblich 2020).

In the brain, adult neuronal progenitor cells (NPCs) can divide and differentiate into various types of neural cells throughout life, contributing to the brain's plasticity and repair after an

injury or illness (Homem, Repic, and Knoblich 2015). For example, neurons and glia can be formed from NPCs, allowing researchers to better understand the mechanisms involved in neuronal differentiation and neurodegenerative disease development. Generating iPSC-derived brain cells from disease patients and aged-matched controls also provides a more translatable model for diseases. Following differentiation, neurons derived from AD patient iPSCs, and from iPSCs carrying FAD mutations, generally exhibit AD-related phenotypes such as elevated Aβ production and increased phosphorylated tau levels (Ochalek et al. 2017; Wezyk et al. 2018; Israel et al. 2012). Such hPSCs models will help elucidate pathways of neurodegenerative diseases and contribute to drug discovery.

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Chapter 2. KNOCK-DOWN OF HDAC2 IN HUMAN INDUCED PLURIPOTENT STEM CELL DERIVED NEURONS IMPROVES NEURONAL MITOCHONDRIAL DYNAMICS, NEURONAL MATURATION AND REDUCES AMYLOID BETA PEPTIDES

2.1 INTRODUCTION

Epigenetic dysregulation is a feature of many neurologic disorders, leading to aberrant gene expression that affects cellular metabolism and function (Lardenoije et al. 2015). Histone acetylation and deacetylation is a common epigenetic modification that can regulate gene expression by controlling chromatin structure. The extent of acetylation is regulated by balanced actions of histone acetyltransferases (HATs) and histone deacetylases (HDACs), regulates integral neuronal functions such as synaptic plasticity (Gräff et al. 2014, 2012; Guan et al. 2009; Penney and Tsai 2014; Yamakawa et al. 2017) and is important in neurodevelopment . Imbalance of histone acetylation and deacetylation contributes to neuronal dysfunction in neurodegenerative disease (Panikker et al. 2018; Bonnaud, Suberbielle, and Malnou 2016).

In particular, histone deacetylase 2 (HDAC2) is an abundant HDAC in the brain and has been shown to specifically regulate genes involved in cognition, learning, and memory (Guan et al. 2009; Penney and Tsai 2014). Dysregulation of HDAC2 has been implicated in neurodegenerative disorders including Alzheimer's Disease (AD) (Gräff et al. 2012; Panikker et al. 2018), where it may contribute to cognitive impairment (Penney and Tsai 2014). Knock-down of HDAC2 leads to up-regulation of synaptic gene expression in primary murine neurons (Yamakawa et al. 2017). Recently, we demonstrated that HDAC2 is involved in regulation of neuronal mitochondrial dynamics via the expression of Endophilin-B1 (SH3GLB1) in primary

murine neurons. This work showed that increased HDAC2 sensitized neurons to mitochondrial dysfunction and cell death in neurodegenerative conditions (Wang et al. 2019).

Human induced pluripotent stem cells (hiPSC) are increasingly utilized for in vitro studies of neurologic disorders. Given that epigenetic dysregulation (P. Li et al. 2019; Zusso et al. 2018), mitochondrial dysfunction (Smith et al. 1996; Manczak, Calkins, and Reddy 2011; Cai and Tammineni 2017), and synaptic dysfunction (K. Li et al. 2018) are all implicated in multiple neurodevelopmental and neurodegenerative diseases, we investigated whether modulation of HDAC2 expression would impact neuronal maturation, mitochondrial dynamics and synaptic gene expression in neuronal cells derived from hiPSCs (hiPSC-Ns). Here we show that HDAC2 levels progressively decrease during neuronal differentiation and this inversely correlates with a natural increase in neuron-specific isoforms of Endophilin-B1 (Endo-B1b/c). We demonstrate that small-hairpin RNA (shRNA)-mediated knock-down of HDAC2 promotes Endo-B1b/c expression in hiPSC-derived cortical neurons, confirming that the regulation of Endo-B1b/c by HDAC2 in human neurons. We then dissect the roles of HDAC2 and Endo-B1b/c in human neurons using knock-down and overexpression experiments to demonstrate that both knock-down of HDAC2 and overexpression of Endo-B1c promote mitochondrial elongation and protect neurons from cytotoxic stress but only knock-down of HDAC2 influences the expression of genes involved mitochondrial gene expression and mitochondrial respiration. Finally, we show that reduction in HDAC2 in hiPSC-Ns decreases levels of Amyloid beta (Aβ), the cleavage product of the amyloid precursor protein (APP) implicated in neurotoxicity in AD. Our data confirm, in a human neuronal cell model, the role of HDAC2 in modulating neuronal synaptic gene expression, and also implicates several pathways in which HDAC2 modulates

mitochondrial dynamics and physiology in hiPSC-Ns. HDAC2 inhibition may represent a therapeutic strategy for AD and other neurodegenerative disorders.

2.2 RESULTS

2.2.1 *Expression of HDAC2 and Endophilin-B1 (Endo-B1) Isoforms in hiPSC-Derived Neurons*

Our previous work demonstrates a role for HDAC2 in regulation of mitochondrial dynamics in primary mouse cortical neurons (Wang et al. 2019). While class I HDACs, HDACs 1 and 2, are required for neuronal specification from neural progenitor cells (Montgomery et al. 2009) and control synapse function and maturation (Akhtar et al. 2009) in mice, HDAC2 expression and regulation of cellular processes in human neurons has not yet been examined. To begin to understand the role of HDAC2 in human neurons, we differentiated cortical neurons from well-characterized hiPSC lines (Gore et al. 2011; Woodruff et al. 2013; Young et al. 2015, 2018) following our standard protocols (Young et al. 2018; Knupp et al. 2020; Rose et al. 2018) (Schematically depicted in Figure 1A). We harvested protein lysates from pluripotent stem cells (hiPSCs), neural progenitor cells that have not yet been directed to a neuronal lineage (NPCs), and neuronally differentiating NPC cultures at week 1, week 2 and week 3 time points. We observed that HDAC2 protein expression is present at all time points, with higher levels in neural progenitor cells that decline as neuronal differentiation proceeds (Figure 1B,C). Interestingly, endogenous expression of all class I HDACs (HDAC 1, 2, and 3) decreased with neuronal differentiation (Supplementary Figure S1). This is consistent with work showing HDAC expression in neural progenitor cells in the developing mouse cortex (Guan et al. 2009). Our previous work in mouse primary cortical neurons demonstrated that expression of HDAC2, but not other class I HDACs, negatively impacts the expression of Endophilin-B1 (Endo-B1), a

multifunctional protein involved in mitochondrial dynamics (Wang et al. 2019). In neurons, Endo-B1 is alternatively spliced yielding neuron-specific Endo-B1b and Endo-B1c as major isoforms relative to the ubiquitously expressed Endo-B1a (Wang et al. 2014). Both of the neuron-specific are neuroprotective with Endo-B1c showing stronger activity in attenuating apoptotic cell death and causing mitochondrial elongation in mouse cortical neurons (Wang et al. 2014). We therefore examined endogenous levels of Endo-B1 isoforms in neuronally differentiating cultures. As the cultures differentiate, we observed an increase in the neuron-specific isoforms of Endo-B1, which co-migrate on a Western blot (designated as Endo-B1b/c), compared to the ubiquitous isoform Endo-B1a, which migrates at a lower molecular weight (Figure 1B,D). We next examined the expression of HDAC2 and Endo-B1 isoforms specifically in differentiating neuronal cells compared with hiPSCs and NPCs. To this end, we enriched our cultures for neurons at each week of neuronal differentiation using magnetic bead sorting according to a modification of previously published protocols (Israel et al. 2012; Yuan et al. 2011). We observed that HDAC2 mRNA expression is dynamically controlled as hiPSCs differentiate toward neuronal lineages (Figure 1E). As the neuron-specific Endo-B1 protein isoforms cannot be separated on Western blot, we designed primers to specifically detect Endo-B1a, Endo-B1b, and Endo-B1c mRNA isoforms and observed strong increases in Endo-B1b and Endo-B1c mRNA during neuronal differentiation with little change observed for Endo-B1a (Figure 1F). The neural-specific splicing factor SRRM4 has recently been implicated in alternative splicing of Endo-B1 pre-mRNA, favoring the generation of Endo-B1b and EndoB1c isoforms over Endo-B1a (Raj et al. 2014). We therefore analyzed SRRM4 levels during neuronal differentiation and observed that endogenous SRRM4 is highly upregulated at week 1 of neuronal differentiation (Figure 1G), corresponding to the appearance of Endo-B1b and Endo-

B1c isoforms at this stage (Figure 1B,F). While SRRM4 levels decline towards the end of the three-week neuronal differentiation, SRRM4 mRNA levels are still significantly increased compared with expression in NPCs and hiPSCs (Figure 1G).

2.2.2 *HDAC2 Knock-Down Influences Neuronal Gene Expression*

Previous studies have implicated HDAC2 as a regulator of synaptic and cognitive gene expression (Gräff et al. 2012) and shown that HDAC2 knock-down (HDAC KD) in mouse primary neurons leads to increases in synaptic gene expression (Yamakawa et al. 2017). As we observed a natural decrease in HDAC2 levels as our hiPSC-Ns differentiate and mature, we tested whether an experimentally evoked decrease in HDAC2 in differentiated hiPSC-Ns would further enhance expression of neuronal genes. In these experiments we differentiated neurons for three weeks and enriched by bead sorting. We transduced neurons with a lentivirus carrying a shRNA against HDAC2 and observed a strong decrease in HDAC2 mRNA (Figure 2A). In these HDAC2 KD cells we observed a significant increase in TBR1 (Figure 2B), a transcription factor expressed in deep layer cortical neurons (Bedogni et al. 2010), suggesting that KD of HDAC2 promotes cortical neuron identity in vitro. In concordance with previous studies (Yamakawa et al. 2017), we observed a significant increase in the mRNA expression of key synaptic genes: SYNGR3, PSD95, SHANK2 and SHANK3 (Figure 2C–F), suggesting that HDAC2 is a repressive regulator of synaptic gene expression in human neurons as well.

2.2.3 *HDAC2 Knock-Down Upregulates the Expression of Endo-B1b/c and SRRM4*

The increasing Endo-B1b/c expression accompanied by declining HDAC2 expression during neuronal differentiation (Figure 1) suggests that the drop in HDAC2 may be promoting Endo-B1b/c expression. We next tested whether HDAC2 KD further increased neuronal

isoforms of Endo-B1. We infected differentiated and enriched neurons with the HDAC2 shRNA lentivirus and observed a significant decrease in HDAC2 protein and increase in the Endo-B1b/c protein as compared to Endo-B1a (Figure 3A–C). We also observed a further increase in SRRM4 mRNA (Figure 3D) and increase in the Endo-B1b and Endo-B1c mRNA splice isoforms (Figure 3E).

2.2.4 *HDAC2 and Endo-B1c Expression Influences Mitochondrial Length*

Enlarged Impaired mitochondrial dynamics and function is a hallmark of neurodegeneration in multiple models (P. A. Li, Hou, and Hao 2017; Grimm and Eckert 2017). Chemical pan-HDAC inhibitors have been shown to induce mitochondrial elongation (Lee et al. 2012). Therefore, we first examined whether HDAC2 KD induces similar changes in mitochondrial shape in human neurons. We analyzed hiPSC-Ns stained with MitoTracker to measure mitochondrial length in the neurites and observed a significant increase in mitochondrial length in HDAC2 KD neurons compared to viral controls (Figure 4A,B). Modulation of HDAC2 changes neuronal mitofusin 2 (MFN2) and mitochondrial fission factor (MFF) expression in mouse primary neurons (Wang et al. 2019). Consistently, we observed that while HDAC2 KD only resulted in a small increase in MFN2 mRNA, it caused a marked decrease in MFF mRNA (Figure 4C,D). At the protein level, MFN2 protein levels were not different in HDAC2 KD neurons whereas MFF protein levels were significantly reduced (Figure 4E–G). Our results suggest that mitochondrial elongation apparent in HDAC2 KD neurons is mediated, at least in part, by a reduction in MFF levels.

Neuron-specific isoforms of Endo-B1 can also influence neuronal mitochondrial dynamics and previous work shows that Endo-B1c had the strongest effect on mitochondrial

length in mouse neurons (Wang et al. 2014). Therefore, we used a lentivirus to infect hiPSC-Ns with a construct that specifically overexpresses the Endo-B1c isoform and analyzed neuritic mitochondrial length. We document strong Endo-B1c overexpression (Figure 5A) and a significant increase in mitochondrial length in neurites (Figure 5B,C). However, overexpression of Endo-B1c did not significantly affect MFF or MFN2 mRNA expression (Figure 5D,E). This is consistent with previous work showing no effect of Endo-B1 KD on these proteins (Wang et al. 2014) and suggests that Endo-B1b/c may affect fusion/fission protein activity in neuronal mitochondria rather than regulating gene expression of these fusion/fission proteins. Taken together, our results indicate that HDAC2 KD induces mitochondrial elongation in human neurons and suggest that it is mediated, at least in part, by increased Endo-B1b/c and decreased MFF expression as a result of HDAC2 KD.

2.2.5 *HDAC2 Expression Influences Neuronal Viability and Mitochondrial Respiration*

Mitochondrial dynamics in cells contributes to viability and metabolism (Schrepfer and Scorrano 2016). EndoB1c has been shown to be anti-apoptotic in neuronal cells while promoting mitochondrial elongation (Wang et al. 2014), so we hypothesized that HDAC2 KD would also be neuroprotective due to the increased expression of Endo-B1c. We knocked down HDAC2 or overexpressed Endo-B1c in hiPSC-Ns and challenged them with the cytotoxic agent camptothecin, a DNA topoisomerase I inhibitor that induces p53-dependent neuronal apoptosis (Uo, Veenstra, and Morrison 2009; Lee et al. 2012). We observed significant protection from camptothecin-induced cell death, as monitored based on caspase-3 activity, in both HDAC2 KD and Endo-B1c overexpression conditions (Figure 6), suggesting that HDAC2 KD can protect against neuronal cell death stimuli partly through elevated expression of neuron-specific Endo-B1 isoforms influencing mitochondrial elongation. Similarly, mitochondrial dynamics may

influence respiration. Overexpression and knock-down of MFN2 has been shown to increase and decrease mitochondrial respiration, respectively and in hiPSC-Ns knock-down of MFN2 has been shown to decrease mitochondrial bioenergetics (Fang et al. 2016) [41]. Inhibition of fission can also impact mitochondrial energy production (Valenti et al. 2017). We measured oxygen consumption rate (OCR), which is an indicator of mitochondrial respiratory activity, in HDAC2 KD neurons using a Seahorse Bioscience XF96 analyzer. We observed a significant increase in basal and maximal OCR and in the spare respiratory capacity in HDAC2 KD neurons (Figure 7A–D). We next tested whether overexpression of Endo-B1c had an effect on mitochondrial respiration but found no significant difference in any of the OCR parameters (Figure 7E–G). An increase in mitochondrial biogenesis could explain the increase in respiration we observe in HDAC2 KD cells, however we did not detect a difference in mtDNA copy number in either HDAC2 knock-down neurons or Endo-B1c overexpressing neurons (Figure 7H). Together, these data suggest that decreasing HDAC2 levels in neurons may enhance mitochondrial respiration by regulating expression of genes that directly regulate metabolism.

2.2.6 *HDAC2 Knock-Down Reduces Amyloid Beta (Aβ) 1–40 and 1–42 Peptides*

Previous studies have demonstrated that HDAC2 expression is specifically increased in neurons in the AD brain using immunohistochemical approaches and HDAC inhibition may have therapeutic potential for neurodegenerative disease (Shukla and Tekwani 2020; Gräff et al. 2012). One of the neuropathological hallmarks of AD is senile plaques comprised of Aβ peptides generated by amyloidogenic cleavage of APP. Aβ peptides can be of various lengths with Aβ 1–40 being the most abundant and Aβ 1–42 being more neurotoxic (Schmidt et al. 2009). We tested whether HDAC2 KD influenced Aβ peptides secreted by hiPSC-Ns in the culture media. hiPSCNs from AD patients as well as from non-demented controls show

detectable levels of Aβ (Young et al. 2015, 2018; Israel et al. 2012) and levels of these peptides can be reduced by treatment with various small molecules (Young et al. 2018; Brownjohn et al. 2017; van der Kant et al. 2019). We infected wild-type (WT) hiPSC-Ns and hiPSC-Ns derived from a patient with a duplication of APP (APPDp) (Rovelet-Lecrux et al. 2006), which has been used and characterized in other studies (Israel et al. 2012), with either the control or HDAC2 KD lentivirus. We measured the levels of secreted Aβ peptides in the culture media and observed that in both WT and APPDp hiPSC-Ns, HDAC2 KD reduced the levels of both Aβ 1–40 and Aβ 1–42 (Figure 8). We see a similar magnitude in Aβ peptide reduction from both the control and the APPDp cell lines, suggesting that HDAC2 does not impact this pathway differently based on the presence or absence of a familial AD mutation and thus may be beneficial in several treatment scenarios.

Figure 1

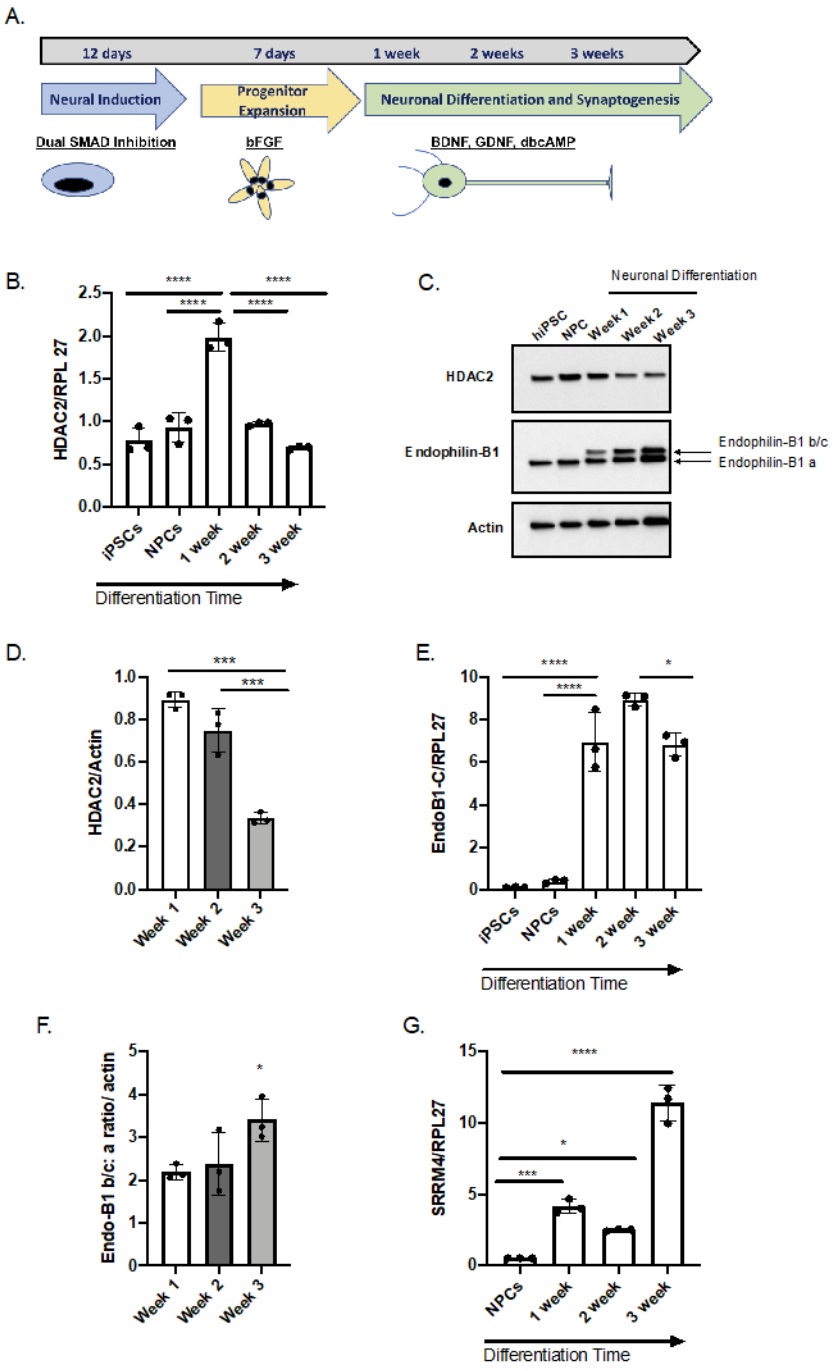

Figure 2.1. **HDAC2 expression is dynamic during neuronal differentiation from hiPSCs and correlates with neuron- specific isoforms of Endophilin-B1, a protein that influences mitochondrial dynamics. (A)**. Schematic of neuronal differentiation protocol from hiPSCs. Arrows indicate time points when NPCs or neurons were enriched by cell sorting. **(B).**

Representative Western blot analysis of endogenous HDAC2 and Endophilin-B1 levels in hiPSCs, NPCs, and during neuronal differentiation. Endo-B1a = ubiquitous isoform, Endo-B1b/c = neuron-specific isoforms. Note: Endo-B1b and Endo-B1c cannot be resolved on Western blot (Wang et al. 2019, 2014, 2015) and are indicated as Endo-B1b/c. **(C)**. Quantification of HDAC2 protein decreases during a three-week neuronal differentiation (N = 3, *** $p < 0.001$ by one-way ANOVA with Tukey's multiple comparisons test). **(D).** Quantification of the ratio of Endo-B1b/c to Endo-B1a protein isoforms during neuronal differentiation (N = 3, * $p < 0.05$ by one-way ANOVA with Tukey's multiple comparisons test). **(E).** Endogenous HDAC2 mRNA harvested from hiPSCs, NPCs, and neuronally enriched cultures harvested at 1, 2, and 3 weeks of differentiation. HDAC2 mRNA expression increases during the first week of neuronal differentiation and then decreases substantially as neurons mature (N = 3, **** $p < 0.0001$ by one-way ANOVA with Tukey's multiple comparisons test). **(F).** Quantification of Endo-B1a, Endo-B1b, and Endo-B1c mRNA isoforms in hiPSCs, NPCs, and neuronally enriched cultures harvested at 1, 2, and 3 weeks of differentiation. Endo-B1b and Endo-B1c mRNA expression is significantly elevated during neuronal differentiation relative to the levels in hiPSCs and NPCs while little change was observed for Endo-B1a mRNA (N = 3, **** $p < 0.0001$ by one-way ANOVA with Tukey's multiple comparisons test) **(G).** Quantification of SRRM4 mRNA in hiPSCs, NPCs, and neuronally enriched cultures harvested at 1, 2, and 3 weeks of differentiation. SRRM4 is highly upregulated during the first two weeks of neuronal differentiation. While levels fall during the third week, SRRM4 transcripts are still at significantly higher levels than in hiPSCs or NPCs (N = 3, ** $p < 0.01$, **** $p < 0.0001$ by one-way ANOVA with Tukey's multiple comparisons test).

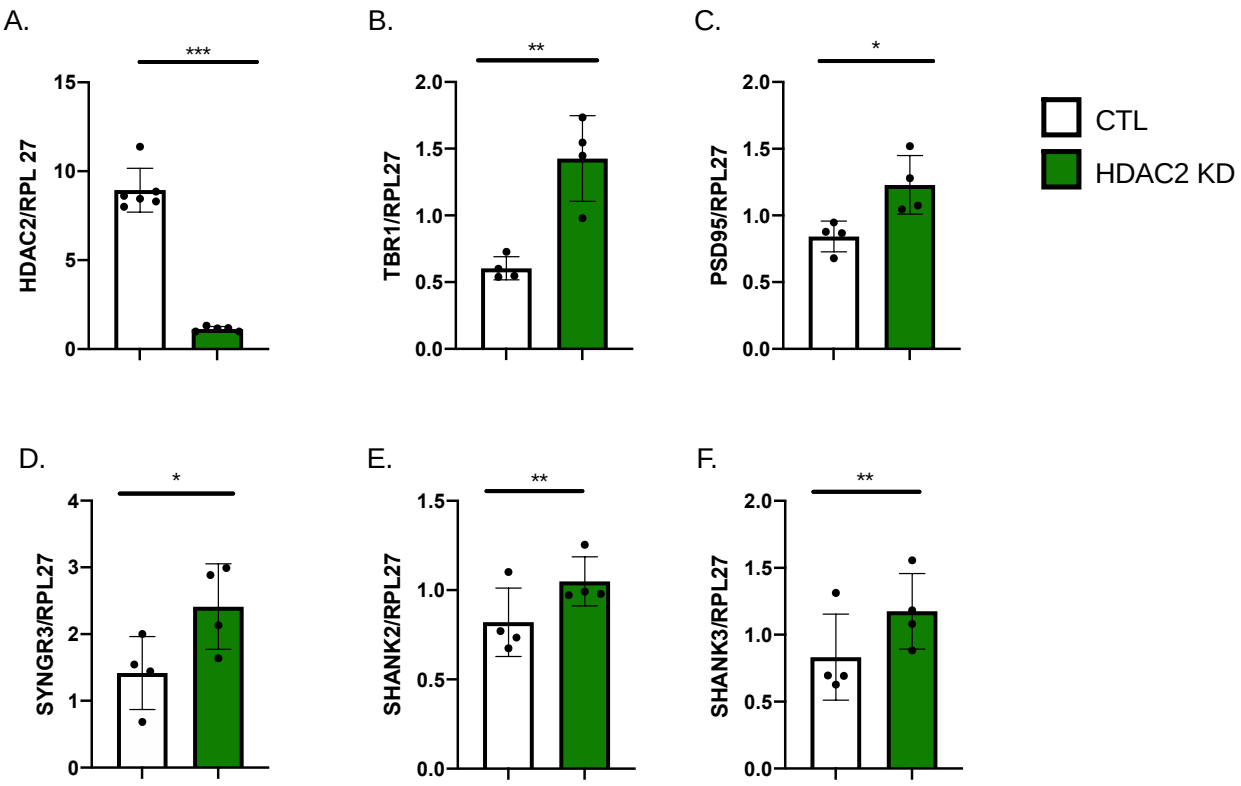

Figure 2.2. **HDAC2 KD in hiPSC-derived neurons influences neuronal and synaptic gene expression APP. (A)**Lentiviral transduction in hiPSC-Ns with an shRNA to HDAC2 significantly reduces HDAC2 mRNA. **(B)**. Knock-down of HDAC2 (HDAC2 KD) increases mRNA of TBR1, a gene that influences cortical neuron identity. **(C–F).** HDAC2 KD increases expression of synaptic genes PSD95 **(C)**, SYNGR3 **(D),** SHANK2 **(E),** and SHANK3 **(F)**. HDAC2 KD vs. CTL neurons (Each dot represents the mean of 4–6 independent experiments, *** $p < 0.001$ by t-test, ** $p < 0.01$ by t-test, * $p < 0.05$ by t-test)

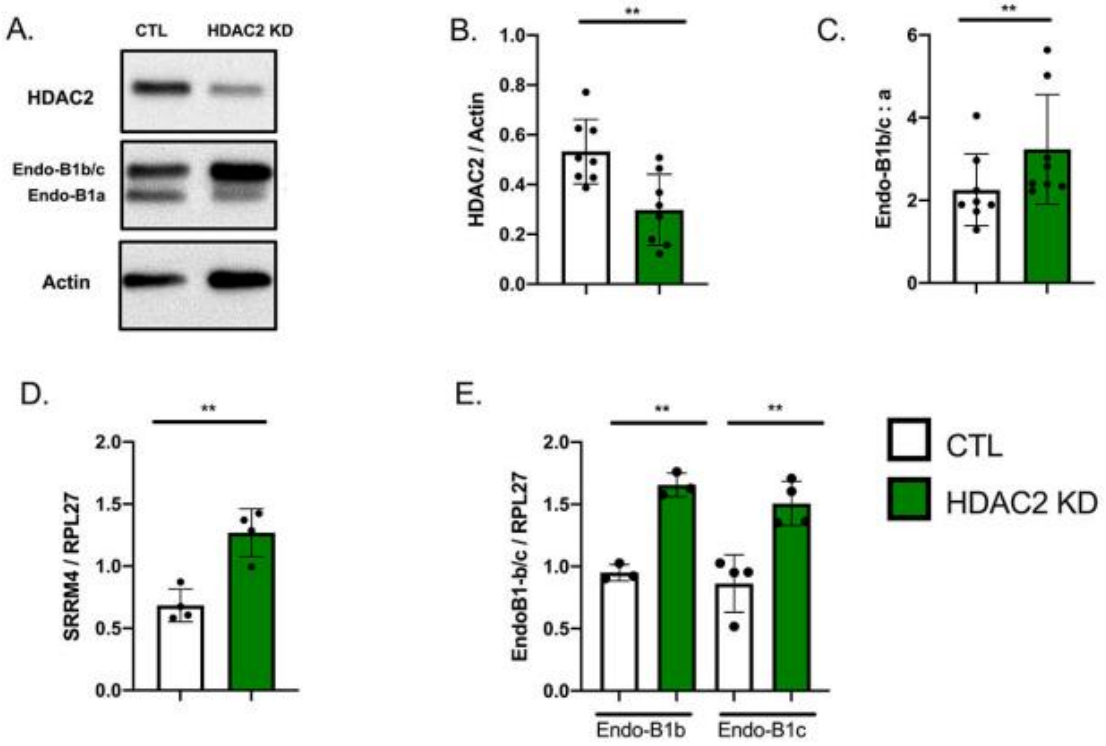

Figure 2.3. **HDAC2 knock-down via shRNA further induces neuronal isoforms of Endo-B1 in hiPSC-derived neurons. (A)**. Representative Western blot demonstrating HDAC2 KD after lentiviral infection and resulting induction in EndoB1b/c isoforms at the protein level. **(B,C).** Quantification of HDAC2 protein and Endo-B1b/c:a isoform ratio in 3-week differentiated neurons transduced with HDAC2 shRNA (N = 8, ** $p < 0.01$ by t-Test). **(D).** HDAC2 KD induces SRRM4 mRNA expression. **(E).** HDAC2 KD induces Endo-B1b and Endo-B1c mRNA expression (Each dot represents the mean of 3–4 independent experiments, ** $p < 0.01$ by t-test).

Figure 4

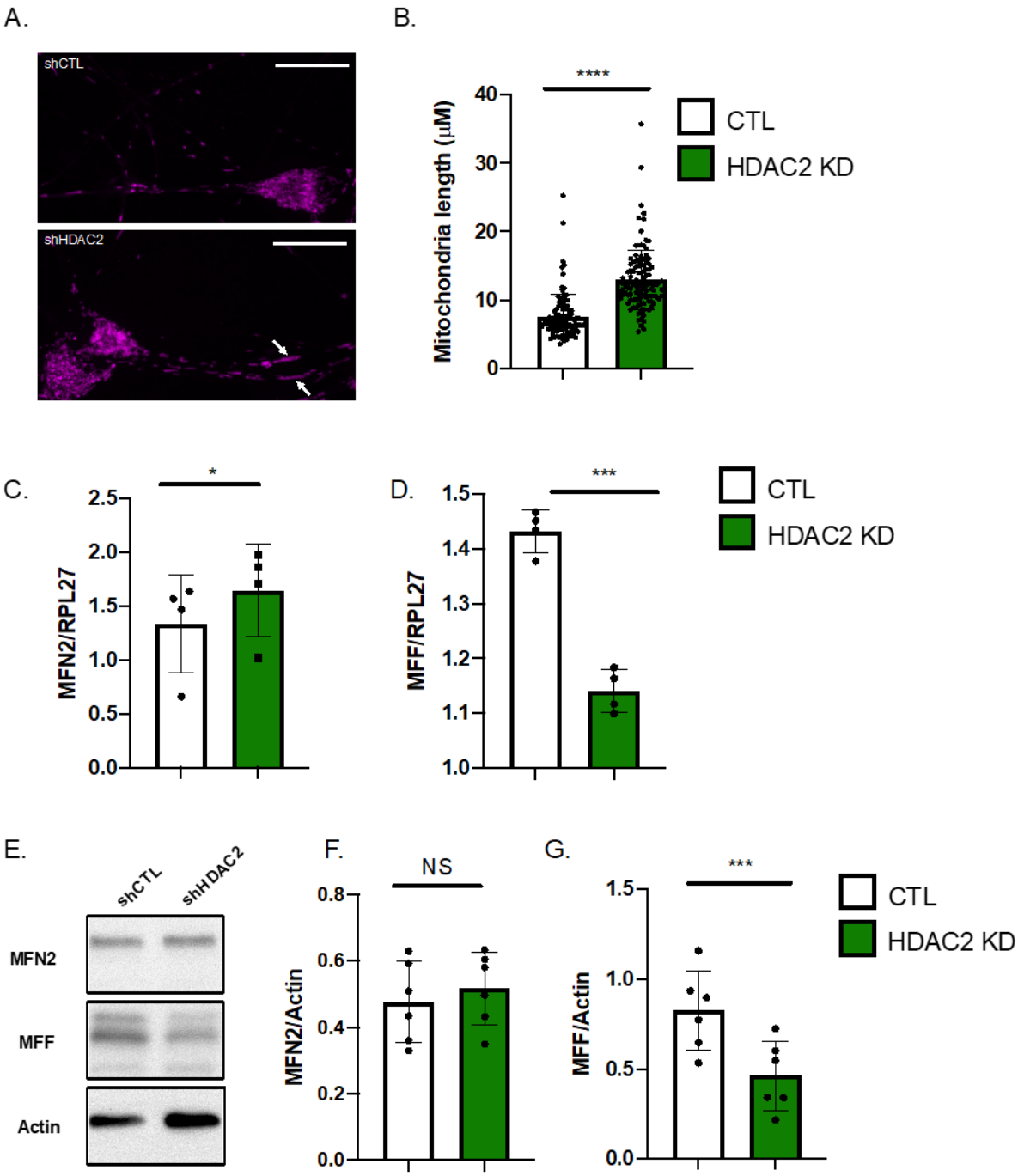

Figure 2.4. **Knock-down of HDAC2 in hiPSC-derived neurons leads to elongated mitochondria in neurites and influences expression of genes involved in mitochondrial dynamics. (A)**WT Representative image of elongated mitochondria visualized with Mitotracker in hiPSC-derived neurons with HDAC2 KD vs. CTL shRNA. **(B).** Quantification of mitochondrial length in neurites (N = 4 independent Mitotracker experiments, 59 control mitochondria and 57

HDAC2 KD mitochondria, **** $p < 0.0001$ by Mann–Whitney U test). **(C).** qRT-PCR analysis of Mitofusin 2 (MFN2) expression in HDAC2 KD (Each dot represents the mean of 4 independent experiments, * $p < 0.05$ by t-test). **(D)**. qRT-PCR analysis of Mitochondrial Fission Factor (MFF) expression in HDAC2 KD (Each dot represents the mean of 4 independent experiments, *** $p < 0.001$ by paired t-test). **(E).** Representative Western blot analysis of MFN2 and MFF protein expression in CTL vs. HDAC2 KD conditions. **(F,G).** Quantification of MFN2 and MFF protein levels: MFN2 protein levels are unchanged in HDAC2 KD (N = 6, NS = not significant by t-test). MFF protein levels are decreased in HDAC2 KD (N = 6, *** $p < 0.001$ by t-test). Scale bar = 20 µm

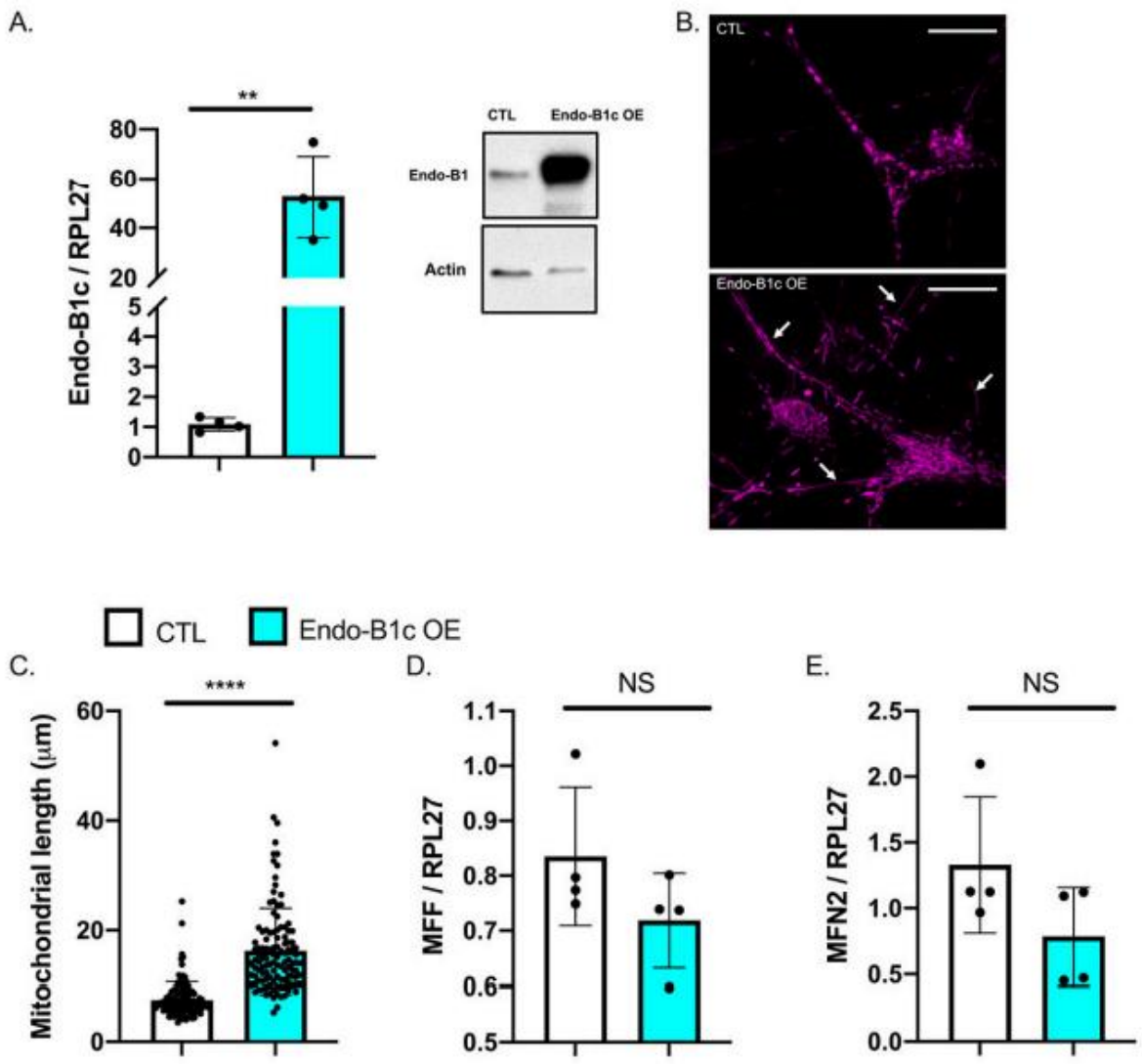

Figure 2.5. **Overexpression (OE) of Endophilin-B1c in hiPSC-derived neurons leads to elongated mitochondria but does not influence MFN2/MFF gene expression. (A).** mRNA quantification of Endo-B1c OE compared to a control OE vector (Each dot represents the mean of 4 independent experiments, ** $p < 0.01$ by t-test). Representative Western blot confirms overexpression of Endo-B1c protein. **(B)**. Representative image of elongated mitochondria visualized with MitoTracker in hiPSC-derived neurons with Endo-B1c OE vs. a control overexpression vector. **(C)**. Quantification of mitochondrial length in neurites (N = 4 independent MitoTracker experiments, 59 control mitochondria, and 62 Endo-B1c OE mitochondria, **** $p < 0.0001$ by Mann–Whitney U test). **(D,E)**. qRT-PCR analysis of MFF **(D)** and MFN2 **(E)** expression in Endo-B1c overexpressing neurons (Each dot represents the mean of 4 independent experiments, NS = not significant by t-test). Scale bar = 20 µm

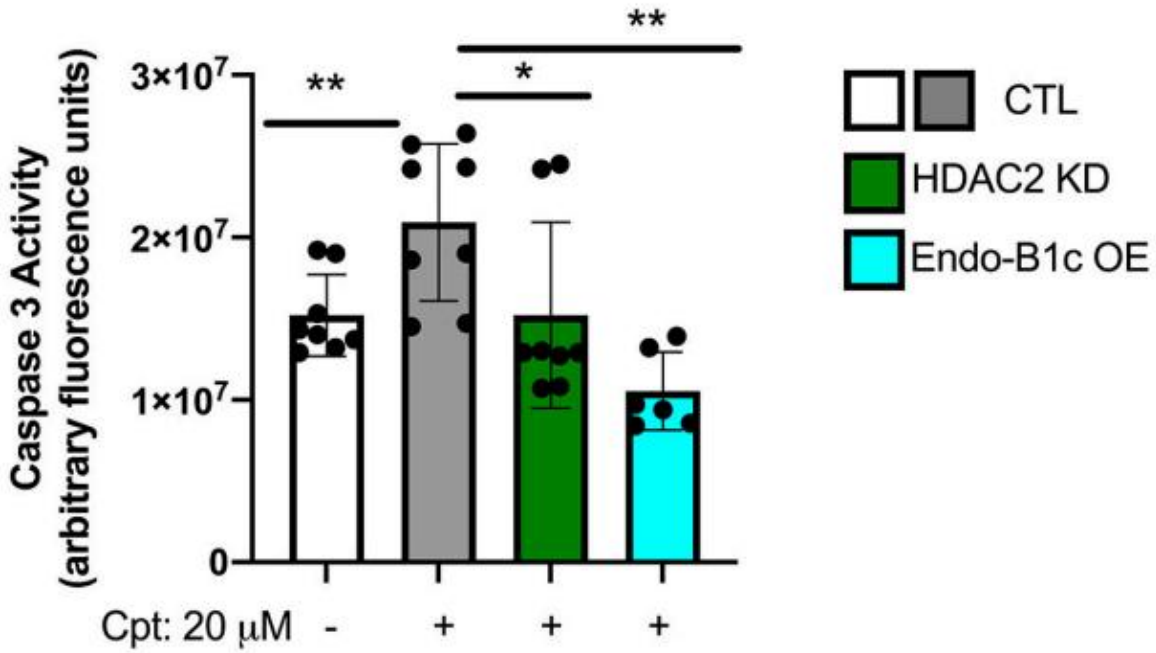

Figure 2.6. **HDAC2 KD and OE of Endo-B1c in hiPSC-derived neurons is protective against neuronal stress.** hiPSC-derived neurons were treated for 24 h with 20 μM camptothecin. Cell death was quantified by measuring caspase-3 activity. Each dot represents a technical replicate of 3–4 independent experiments. Analysis compares all groups to the CTL/untreated bar (white) using a one-way ANOVA with Tukey post hoc multiple comparisons; ** $p < 0.01$; * $p < 0.05$.

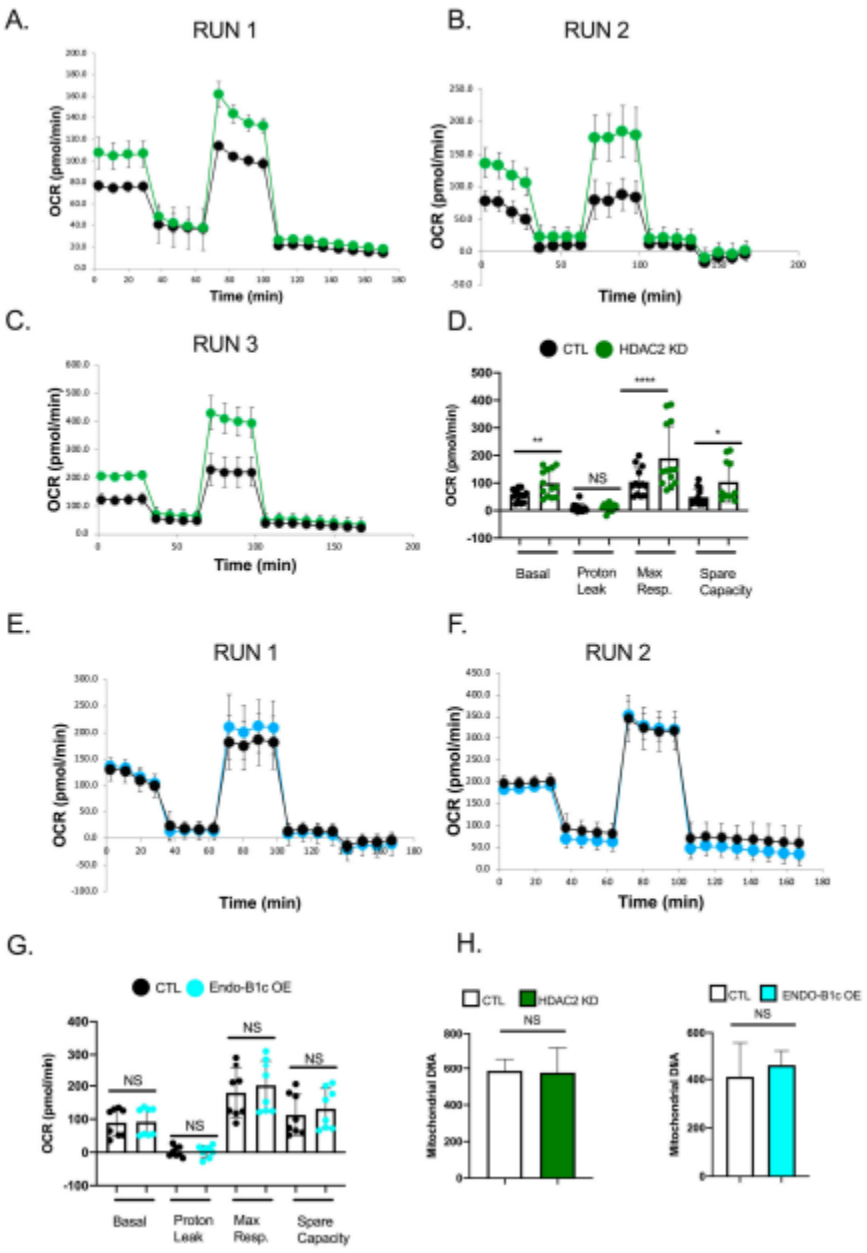

Figure 2.7. **HDAC2 KD but not OE of EndophilinB1-c improves mitochondrial respiration in hiPSC-derived neurons. (A-C)** Plots of individual Seahorse assays measuring the oxygen consumption rate (OCR) of hiPSC-Ns with HDAC2 KD compared to a control virus. **(D).** Compilation of the three independent Seahorse assays (shown in **A–C**) shows significant increases

in basal respiration, maximum respiration (Max resp.), and spare capacity. Each dot represents a technical replicate within each independent assay and data are analyzed using a one-way ANOVA with Tukey post hoc multiple comparisons; * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$. (**E,F**). Plots of individual Seahorse assays of hiPSC-Ns with EndoB1-c overexpression compared to a control virus. (**G**). Compilation of the two independent Seahorse assays (shown in **E,F**) shows no significant changes in basal respiration, maximum respiration (Max resp.), proton leak, or spare capacity. Each dot represents a technical replicate within each independent assay and data are analyzed using a one-way ANOVA with Tukey post hoc multiple comparisons; NS = non-significant. (**H**). Analysis of mitochondrial DNA copy number in hiPSC-Ns with HDAC2 KD or Endo-B1c OE. No significant difference was found in mitochondrial copy number in either condition when compared to a control virus. NS = non-significant by t-test.

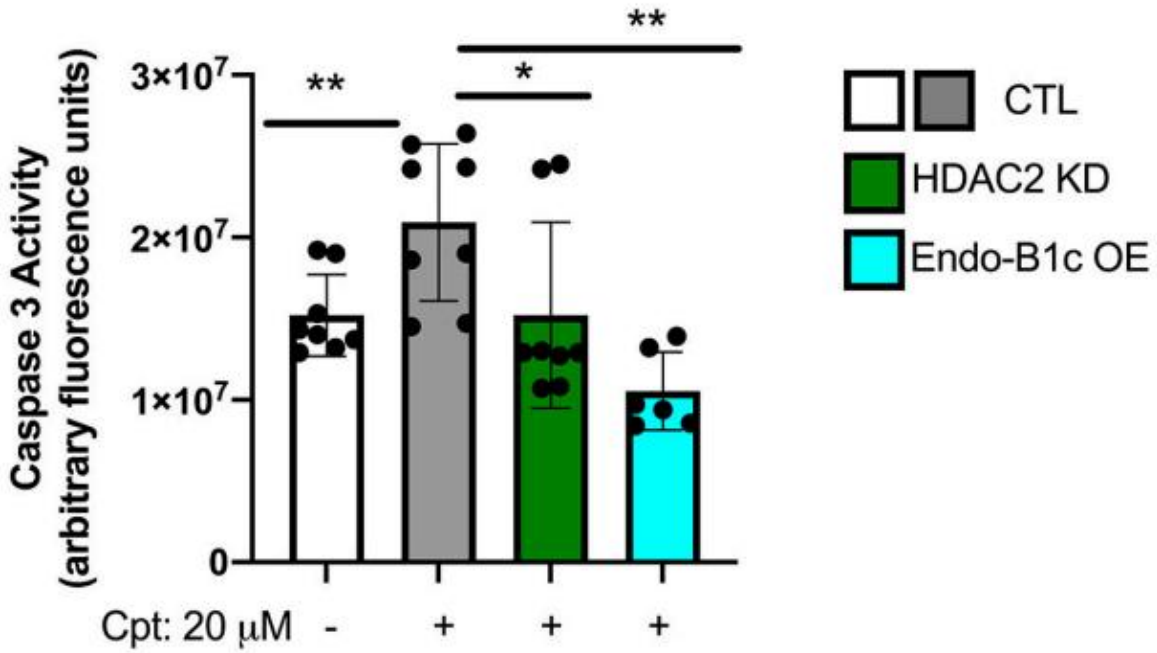

Figure 2.8 **Knock-down of HDAC2 reduces Aβ peptides in hiPSC-Ns. Aβ peptides (1–40 and 1–42) secreted in the media from both WT and APPDp hiPSC-Ns were reduced under HDAC2 knock-down conditions.** The data represent two independent differentiations, CTL vs. HDAC2 KD analyzed by t-test ** $p < 0.01$; * $p < 0.05$.

2.3 DISCUSSION

Acetylation and deacetylation of histones is critical for regulating gene expression and is essential to normal neuronal development and function with dysregulated acetylation/deacetylation contributing to development of neurodegenerative conditions. Expression of the epigenetic regulator HDAC2 is altered in neurodegenerative diseases such as Alzheimer's disease and this may influence expression of genes related to cognition (Gräff et al. 2012). Previously, we demonstrated that HDAC2 regulates expression of neuron-specific isoforms of Endo-B1, a protein that confers neuroprotection and promotes mitochondrial elongation, uncovering a novel role of HDAC2 in regulation of mitochondrial function (Wang et al. 2019). In the current study, we sought to understand how HDAC2 expression influences hiPSC-N maturation and mitochondrial size and function. We first assessed HDAC2 expression during neuronal differentiation from hiPSCs and demonstrated progressively decreasing levels of HDAC2 mRNA and protein with time. HDAC2 is a class I HDAC that is important for neurodevelopment (D'Mello 2019). Studies in olfactory receptor neurons in the olfactory epithelium of mice showed that HDAC2 is highly expressed in early post-mitotic neurons, but not glia, and is downregulated during neuronal maturation (MacDonald, Gin, and Roskams 2005; MacDonald and Roskams 2008). Other studies show that HDAC2 expression is important for silencing neural progenitor transcripts during adult neurogenesis in the mouse (Jawerka et al. 2010). Together, these studies indicate that HDAC2 expression is dynamic in differentiating and maturing neurons. In our study, we detect HDAC2 expression at mRNA and protein levels at all stages of the cells we studied (pluripotent-hiPSCs, neural progenitor cells-NPCs, and differentiating neurons). Interestingly, HDAC2 levels appear to increase one week after neuronal induction from NPCs and then decrease as neurons further differentiate and mature, however

there is still measurable expression of HDAC2 in differentiated neurons on par with the level in hiPSCs. Future work looking at expression of class I HDACs in differentiating neurons and glial cells will be important in determining the complex roles these HDACs play in human neural development.

As we noted a decline in HDAC2 levels as hiPSC-Ns matured during neuronal differentiation, we hypothesized that the decline in HDAC2 levels may facilitate expression of genes that support neuronal function. Previous work in mouse primary neurons showed that knock-down of HDAC2 induces expression of synaptic genes by directly regulating histone acetylation at the promoter (Yamakawa et al. 2017), which is complementary to data suggesting that upregulation of HDAC2 negatively affects expression of genes involved in cognition (Gräff et al. 2012). Consistent with these results, using an shRNA approach to further decrease HDAC2 levels in our differentiated hiPSC-Ns we revealed that decreased HDAC2 levels lead to increased expression of pre- and post-synaptic genes. Although our results cannot rule out an indirect effect of HDAC2 on genes we did not measure, these previous and current findings suggest that decreasing HDAC2 levels in neurons, including human neurons, may promote neuronal maturation and function and that lowering HDAC2 levels may be a strategy to restore normal function in neurologic disorders.

Previously we reported that HDAC2 regulates expression of Endo-B1, a multifunctional protein involved in mitochondrial dynamics. Specifically, we showed that decreasing HDAC2 levels in mouse neurons inversely elevates neuron-specific and neuroprotective Endo-B1 isoforms (Wang et al. 2019). Neuron-specific isoforms of Endo-B1 promote neuronal survival and their expression in the brain is reduced in mouse models and human models of AD and mouse models of stroke (Wang et al. 2014, 2015). We observed an increase in neuron-specific

isoforms, Endo-B1b/c, as our human neurons differentiate and mature, consistent with the decreasing HDAC2 expression concurrently observed. Prompted by previous reports implicating the neuron-specific splicing factor SRRM4 in the alternative splicing of Endo-B1 (Raj et al. 2014; Gan et al. 2018), we examined whether changes in SRRM4 expression correlate with the appearance of Endo-B1b/c isoforms. We found that SRRM4 mRNA expression is indeed induced upon neuronal differentiation and further demonstrated that SRRM4 mRNA is upregulated by HDAC2 KD in differentiated neurons, suggesting that SRRM4 gene expression may be negatively regulated by HDAC2 in human neurons. Thus, our data suggest that lowering HDAC2 levels may promote expression of SRRM4 facilitating neuron-specific splicing of Endo-B1, which in turn can allow the resulting neuron-specific Endo-B1b/c isoforms to promote mitochondrial elongation. As expected, overexpression of Endo-B1c in hiPSC-Ns had a significant effect on mitochondrial elongation as well.

While knock-down of HDAC2 increases mitochondrial elongation likely through the induction in Endo-B1c, we also examined expression of MFN2 and MFF, molecules that promote fusion and fission of mitochondria, respectively. Our previous work showed that HDAC2 overexpression increases MFF expression while decreasing MFN2 expression in mouse neurons (Wang et al. 2019). Consistently, HDAC2 KD in differentiated hiPSC-Ns conversely induced a significant decrease in MFF mRNA and protein although no significant effect was observed for MFN2 mRNA. Separately, we confirmed that Endo-B1c overexpression has no effect on MFF or MFN2 mRNA expression, ruling out any transcriptional activity of Endo-B1c, consistent with the reported cytosolic and/or mitochondrial localization of Endo-B1 (Karbowski, Jeong, and Youle 2004). Endo-B1c may thus have a more physical role in elongation of mitochondria. Indeed, recent work shows that Endo-B1 can regulate the mitochondrial inner

membrane through an interaction with prohibitin-2 (Cho et al. 2019). Taken together, our data suggest that modulation of HDAC2 in hiPSC-Ns influences mitochondrial dynamics, in part, through regulation of the expression of fusion and fission proteins, which includes alternative splicing-regulated production of the net fusion-promoting Endo-B1b/c isoforms (with the assistance of concomitantly regulated expression of SRRM4) and transcriptional regulation of MFF gene.

In addition to mitochondrial elongation, increased mitochondrial biogenesis is reported in terminally differentiated mouse cortical neurons (Agostini et al. 2016). In human pluripotent stem cell differentiations, cells undergo a shift from glycolysis in neural progenitors to oxidative phosphorylation in differentiating neurons (Zheng et al. 2016). We therefore decided to examine the effects of HDAC2 KD and the resulting upregulation of Endo-B1b/c on mitochondrial respiration and biogenesis in hiPSC-Ns. Analyses using a Seahorse Flux analyzer demonstrated that HDAC2 KD evokes a significant increase in OCRs representing the basal respiration, the maximal respiration and the spare respiratory capacity. This suggests that lowering levels of HDAC2 improves multiple aspects of mitochondrial physiology towards, for instance, more neuronally mature and thus more oxidative modes of metabolism and augmented bioenergetic capacity to confer increased resistance to stress. Interestingly, a similar action of HDACs has been reported in HL-1 cells derived from mouse atrial cardiac muscle, where HDAC inhibition with a class I HDAC-specific inhibitor, MPT0E014, improves mitochondrial OCR following TNF-alpha treatment to model heart failure (Lkhagva et al. 2018). These findings suggest that reducing HDACs may be a conserved process that could benefit mitochondrial bioenergetics across many different tissues. Interestingly, overexpression of Endo-B1c did not significantly affect these respiratory parameters. This indicates that the improved

respiratory function in HDAC2 KD neurons is not solely a result of increased mitochondrial length. The absence of an increase in oxidative phosphorylation despite mitochondrial elongation induced by Endo-B1c overexpression suggests that these two functions are not always dependent on each other. Indeed, a carboxy-terminal truncation of MFN2 has been shown abrogate its mitochondrial fusion capacity but was still able to induce an increase in mitochondrial membrane potential and stimulate glucose oxidation (Pich et al. 2005). Finally, we also tested whether the changes in mitochondrial respiration in HDAC2 KD cells were due to increases in mitochondrial biogenesis, however we did not see significant differences in mtDNA copy number in either HDAC2 KD or Endo-B1c OE cells.

As decreased HDAC2 levels induce Endo-B1c expression, which is known to be anti-apoptotic in neurons, we tested whether HDAC2 KD or Endo-B1c overexpression would protect hiPSC-Ns against a neurotoxic insult. Both HDAC2 KD and Endo-B1c overexpression significantly reduced caspase-3 activity induced by camptothecin treatment. These data suggest that lowering HDAC2 levels, either by genetic or pharmacologic means, may be a viable strategy in maintaining or restoring viability of human neurons during disease conditions and that forced expression of Endo-B1c could substitute such HDAC2 manipulation.

Mitochondrial and synaptic dysfunction are implicated in neurodegenerative disease pathogenesis and strategies to improve these pathways can be considered for novel therapeutic development. Indeed, HDAC inhibition has been suggested as a possible therapeutic pathways for neurodegeneration (Shukla and Tekwani 2020) We tested whether HDAC2 knock-down could improve a cellular phenotype related to AD pathology, Aβ secretion into neuronal culture media. In both control and APPDp neurons, knock-down of HDAC2 significantly reduced the levels of secreted Aβ 1–40 and Aβ1–42 peptides. These data suggest that further investigation

into HDAC inhibition, specifically HDAC2 inhibition, as a therapeutic strategy for AD is warranted.

In this study, we report a role for endogenous HDAC2 during human neuronal differentiation. Our data suggest a repressive regulation of the neuron-specific splicing factor SRRM4 by HDAC2 and highlights Endo-B1b/c isoforms as novel functional contributors to and markers of human cortical neuronal differentiation. Using lentiviral knock-down and overexpression approaches we further confirm previous work in mice demonstrating that HDAC2 acts repressively on synaptic gene expression and we solidify the finding that HDAC2 regulates genes involved in mitochondrial bioenergetics and dynamics by showing, for the first time, this effect in hiPSC-Ns. Finally, we show a significant effect of HDAC2 on neuronal mitochondrial respiration and neuroprotection. Our data support the idea that manipulation of HDAC2 may be beneficial in the treatment of neurological diseases.

2.4 MATERIALS AND METHODS

2.4.1 *Cell Culture and hiPSC Neuronal Differentiation*

These experiments represent data obtained from two well characterized control hiPSCs lines (CV and WTC11) (Levy et al. 2007; Miyaoka et al. 2014). The data showing reduction in Aβ in FAD cells was obtained from a cell line with an APP duplication (Israel et al. 2012). The CV line was generated at the University of California, San Diego and was transferred to Dr. Young via a Material Transfer Agreement. The APPDp cell lines was generated at the University of California, San Diego and was transferred to Dr. Young via a Material Transfer Agreement. The WTC11 line was obtained from Dr. Carol Ware at the University of Washington's Ellison Stem Cell Core. hiPSCs were cultured under feeder-free conditions and differentiated to neural progenitor cells (NPCs) following published protocols utilizing dual SMAD inhibition and after

12 days NPCs were purified using sorting for CD184+/CD24+ populations as previously described [30,59,60]. Neuronal differentiation of NPCs to cortical neurons was performed as we have previously published (Young et al. 2015; Knupp et al. 2020; Rose et al. 2018). Briefly, NPCs were seeded at 10 × 106 per 10 cm plate and differentiated for three weeks in the presence of GDNF (20 ng/mL PeproTech, Rocky Hill, NJ, USA), BDNF (20 ng/mL PeproTech, Rocky Hill, NJ, USA) and dbcAMP (250 ug/mL Sigma, St. Louis, MO, USA). To analyze cultures enriched in neurons, differentiated cultures were dissociated into a single cell suspension using Accutase (Innovative Cell Technologies, San Diego, CA, USA) and incubated with antibodies against CD184-PE and CD44-PE (BD Biosciences, San Jose CA, USA). Anti-PE magnetic beads (BD Biosciences, San Jose CA, USA) were added, and complexes were pulled down. The neuronally enriched supernatant was re-plated in a modification of the sorting protocol published by Yuan et al. (Yuan et al. 2011) and used in our previous publications (Young et al. 2018; Knupp et al. 2020; Rose et al. 2018).

2.4.2 *Lentivirus Treatment*

Lentiviruses carrying a control shRNA, HDAC2 shRNA, GFP or Endo-B1c plasmids were generated as previously described (Gräff et al. 2012; Wang et al. 2019). For all lentivirus treatments, neurons were differentiated for three weeks and enriched by bead sorting. Four days after enrichment, viruses were added to the cultures and left for three days. Cells were further maintained in virus-free medium for 2 weeks and then harvested or used for assays.

2.4.3 *RNA Purification and qPCR Analysis*

Total RNA was purified from 2×10^5 cells using TRIzol (Life Technologies, Carlsbad, CA, USA) followed by 1st strand cDNA synthetized using the iScript kit (Biorad, Hercules, CA,

USA). Between 5 and 10 ng of cDNA were used in a 4 uL reaction using POWEUP SYBR qPCR mix (Life Technologies, Carlsbad, CA, USA). All primer-sets were run in technical triplicates. Expression was calculated using the 2—ΔΔCt method and genes of interest were normalized to RPL27. qPCR primers designed over exon-exon boundaries using Primer-Blast are below:

hHDAC2	TGAGATTCCCAATGAGTTGCCA	TACTGACATCTGGTCAGACA
hMFN2	CACCCTGATGCAGACGGAAA	TCCATGTACTCGGGCTCTGA
hMFF	CAGCTTCACTAAGACGACAGATAA	TTACCTCTAGCGGCGAAACC
hPSD95	CTCAGGGTCAACGACAGCAT	AAGCCAAGACCTTTAGGCCC
hRPL27	TGAGATTCCCAATGAGTTGCCA	TACTGACATCTGGTCAGACA
hSH3GLB- C	CATGTAAAATGGCTGAAGATTTGG	TGGGCATGTGTACTGCTGAT
hSHANK2	CTGGCGAGCTGGGGTGATTA	TCAATGGGTGTGTCAGCTTTG
hSHANK3	CCTCACCTCACACAGCGATT	CCACCGACTCGAGATACTGC
hSYNGR3	CGTCCTGGGTGTTCTCCATC	CTGCTGATTTGCTGGAAGCG
hSRMM4	ATAGCCCATCGCCTGTCAAG	GCCGGCTTCGAGATTGTTTC

2.4.4 *Western Blot Analysis*

2 × 105 cells were lysed in 50 µL RIPA buffer and protein amount was assessed using BCA assay kit (Thermo Scientific, Waltham, MA, USA). Between 3 and 10 µg of total protein were loaded on to a 4–15% gradient TGX gel (Bio-Rad) and transferred on to a PVDF

membrane (Bio-Rad), which was then treated with 0.4% paraformaldehyde for 30 min to fix proteins. Following blocking with 5% nonfat dry milk, 0.1% Tween 20 and 0.05% thimerosal, membrane was incubated with the primary antibody diluted in 5% bovine serum albumin, 0.1% Tween 20, 0.05% thimerosal and 0.2% NaN3 overnight at 4 ◦C, followed by horse radish peroxidase-conjugated secondary antibody (1:2000, GE Healthcare, Chicago IL, USA) diluted in the blocking buffer. Membrane was then developed using Clarity Western ECL substrate (Biorad, Hercules, CA, USA) and exposed to Hyperfilm ECL (GE Healthcare, Chicago IL, USA). Films were digitally scanned, and band intensity was quantitated using ImageJ and normalized against β-actin.

Antibodies		
HDAC2	Sigma H2663 (2 mg/mL)	1:5000
Bif-1/Endophilin-B1(30A882.1.1)	Novus #NBP2-24733 (0.5 mg/mL)	1:1000
MFN2	Abcam #ab124773 (1.549 mg/mL)	1:2000
MFF	Proteintech #17090-1-AP (45 µg/150 µL)	1:2000
Actin	Sigma A5441	1:2500

2.4.5 *Mitochondrial Length Analysis*

Mitochondria were stained with MitoTracker Red (M7513 Invitrogen/ Thermo Scientific, Waltham, MA, USA) following manufacturer's instructions, and pictures of at least three independent fields per sample were taken on a NIKON A1R confocal system using a 60x objective. Mitochondrial length was quantified by a blinded observer using ImageJ as we have previously described (Wang et al. 2014).

2.4.6 *Caspase 3 Analysis*

Neurons were cultured in a 96 well plate, in replicates, at a concentration of 2 × 105

cells/well, treated with lentiviruses as indicated above (4.2) and were then treated for 24 h with 20 µM camptothecin or with vehicle (DMSO) alone. Cells were then lysed, and a caspase-3 assay was performed according to the manufacturer's protocol (EnzChek caspase-3 Assay, Invitrogen/ Thermo Scientific, Waltham, MA, USA). The intensity of fluorescence was analyzed using an EnVision plate reader (PerkinElmer, Waltham, MA, USA).

2.4.7 *Seahorse Analysis*

Neurons were plated in a Matrigel-coated 96 well Seahorse plate at a density of 2×10^5 cells/well and transduced with lentivirus as described above. The MitoStress protocol in the Seahorse XF96 Flux Analyzer (Agilent Technologies, Santa Clara, CA, USA) was performed two weeks later. An hour before the assay, the culture media was replaced with base media (Agilent Seahorse XF base medium, 103334-100 Agilent Technologies, Santa Clara, CA, USA) supplemented with 25 mM glucose and 1 mM Sodium pyruvate (11360070 Gibco/Thermo Scientific, Waltham, MA, USA). Substrates and select inhibitors of the different complexes were injected during the measurement to achieve final concentrations of oligomycin (2.5 µM), FCCP (1 µM), rotenone (2.5 µM) and antimycin (2.5 µM). The oxygen consumption rate (OCR) values were then normalized with readings from Hoechst staining (HO33342 Sigma-Aldrich, St. Louis, MO, USA), which corresponded to the number of cells in the well.

2.4.8 *Mitochondrial DNA Copy Number Analysis*

Genomic DNA was prepared from samples in TRIzol (Life Technologies Carlsbad, CA, USA) that had previously been used to extract RNA according to manufacturer's protocol. Then, 10 ng of genomic DNA were used per qPCR reaction and each sample was assessed in triplicate for mitochondrial ND1 (F: CCCTAAAACCCGCCACATCT, R:

GAGCGATGGTGAGAGCTAAGGT) and nuclear LPL (F: CGAGTCGTCTTTCTCCTGATGAT, R: TTCTGGATTCCAATGCTTCGA) genes. Nuclear DNA-normalized, relative mitochondrial DNA content is provided by $2 \times 2\Delta CT$ where ΔCT = (nucDNA CT–mtDNA CT).

2.4.9 *Aβ 1–40 and 1–42 Analysis*

Aβ peptides were measured as previously described (Young et al. 2018). Briefly neurons were differentiated as described above in 4.1 and plated at 200,000 cells per well. The neurons were treated with lentiviruses as described above in 4.2. Medium was harvested to measure secreted Aβ peptides, media was run on an Aβ V-plex ELISA plate (Meso Scale Discovery, Rockville, MD, USA) per manufacturer's instructions.

2.4.10 *Quantification and Statistical Analysis*

Data represent two individual cell lines performed in biological and technical replicates. All data were analyzed using GraphPad Prism software v. 8 (GraphPad Software, Inc., La Jolla, CA, USA). For each data set, data were analyzed for normal distribution using the Shapiro–Wilk Test. Normally distributed data were analyzed using parametric statistical tests. For comparisons of more than two groups one-way ANOVA analysis was used with Tukey's post hoc multiple comparisons test. For data in two groups two-tailed t-Tests were used. For non-normally distributed data, non-parametric tests were used. For non-normal data comparing two groups, the Mann–Whitney U test was used. Definition of replicates and all statistical tests used, and p-values are reported in the figure legends.

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Chapter 3. CHARACTERIZING THE ROLE OF HDAC2 IN NEURONAL METABOLISM IN HIPSC-DERIVED NEURONS

3.1 INTRODUCTION

In recent years, researchers have made significant strides in unraveling the complex interplay between neuronal metabolism and epigenetic regulation. One particular focus of the investigation is the metabolic switch that occurs in neurons, shifting from glycolysis (the breakdown of glucose) to oxidative metabolism (the utilization of mitochondrial respiration) under specific physiological and pathological conditions (Zheng et al. 2016; Lujan et al. 2021; Rangaraju, Calloway, and Ryan 2014; Harris, Jolivet, and Attwell 2012). This metabolic switch is not only essential for sustaining neuronal energy demands but also plays a crucial role in orchestrating various cellular processes that underlie neuronal function and plasticity.

HDAC2 is known for its role in modifying chromatin structure and regulating gene expression by removing acetyl groups from histone proteins (Inoue and Fujimoto 1969). By deacetylating histones, HDAC2 can tighten the packaging of DNA, leading to gene silencing and impacting various cellular processes. Studies have shown that during periods of increased neuronal activity, such as learning and memory formation, there is a downregulation of HDAC2 (Fischer et al. 2007; Gräff et al. 2012). This downregulation allows for a more permissive chromatin structure, facilitating the expression of genes necessary for synaptic plasticity and memory consolidation. On the converse, during aging and in diseases such as Alzheimer's Disease, HDAC2 levels are elevated and further repress genes that are necessary for synaptic plasticity and function (Guan et al. 2009; D. Liu et al. 2017).

Interestingly, the metabolic state of neurons appears to be closely tied to the regulation of HDAC2 activity. We reported an increased respiratory profile upon knockdown of HDAC2 in human induced pluripotent stem cells (hiPSC) (Frankowski et al. 2021). Other reports have also shown the ability of HDAC2 to influence various mitochondrial genes (D. B. Wang et al. 2015). In this study, we sought to understand the underlying factors that account for the increased respiratory profile. We employed bulk RNA-seq analysis, metabolomics and phenotypic assays to decipher the gene network and pathways that influence metabolism upon HDAC2 downregulation in hiPSC-derived neurons. Our data shows that knockdown of HDAC2 leads to significant changes in the transcriptomic and metabolic levels of cells. Further probing our datasets, metabolic and neurodevelopmental pathways seem to be the most affected in our data set. With this, we sought to understand the activity of the various mitochondria complexes and found that there is a decrease in complex I (CI) enzymatic activity but a remarkable increase in complex V/ATP Synthase enzymatic activity. The increased respiration may be driven by changes in mitochondrial membrane potential but we realized that was not the case as there was no change to the membrane potential. Neither was there an increase in basal lipid peroxidation, a readout of the amount of reactive oxygen species. Overall, HDAC2 inhibition yield beneficial results in hiPSC through a complex model of pathways and cascade of events. Understanding this intricate relationship between neuronal metabolism and HDAC2 activity holds great promise for uncovering new therapeutic avenues. Further studies will help delineate these molecular pathways.

3.2 RESULTS

3.2.1 *HDAC2 knock-down influences various genes and pathways related to mitochondrial pathways and maturation*

Using CRISPR/Cas9 genome editing techniques, we previously generated hiPSC-derived HDAC2 is involved in various biological processes, including development, differentiation, and cellular homeostasis, and has also been implicated in multiple disease states, including cancer, neurodegenerative disorders, and cardiovascular diseases (Guan et al. 2009; Uo, Veenstra, and Morrison 2009; Lkhagva et al. 2018). Dysregulation of HDAC2 activity has been associated with aberrant gene expression patterns and contributes to disease pathogenesis. However, the exact pathways leading to these effects have not been well characterized. Seeking to obtain a holistic view of transcriptomic alterations including the various pathways and gene clusters that are altered upon knockdown of HDAC2, we performed a bulk RNA sequencing (RNA-Seq) experiment with two wildtype and AD cell lines.

Strikingly, we observed that there were significantly more downregulated genes in HDAC2 knockdown neurons than upregulated ones in both wild-type and AD lines (Fig. 1A,1B,1C). With a more open chromatin structure upon HDAC2 KD, we anticipated the opposite. From the G0-term analysis and pathway enrichment studies, we had mitochondrial and metabolic pathways among the most significantly upregulated pathways (Fig 1E). By examining the genes of the various mitochondrial complex, we did see both increases and decreases in the expression level of the various genes (Fig 1F). We further confirmed the expression of individual subunits of the mitochondrial complex via an antibody cocktail and saw a decrease in most subunits of the mitochondria complexes (Fig 1G). We also observed an increase in genes related to neuron maturation and development. This observation again confirmed our prior studies where

we showed endogenous decline of HDAC2 as neurons mature as well as the increased respiratory profile via the Seahorse assay. The most downregulated pathways pointed to various cell cycle pathways (Fig 1D). Indeed, with neurons being post-mitotic cells, there is a cell cycle arrest as neurons mature. We are still employing various means to probe the dataset and tease out differences in diseases versus wild-type neurons to identify novel pathways that can be harnessed for AD therapy and advance our knowledge of various biological processes and AD.

3.2.2 *HDAC2 knock-down influences metabolomic changes*

While the Seahorse assay provides a general overview of the oxygen consumption rate, it does not tell which key metabolites are affected upon HDAC2 knockdown. To understand this, we ran a targeted metabolomic of 300+ aqueous metabolites in both positive and negative ionization modes from 42 KEGG–defined metabolic pathways, along with ~30 stable-isotope labeled internal standards for concentration determinations. We had a great spread between up and adown regulated metabolites (Fig 2A). Some downregulated metabolites include 2-Hydroxyglutarate, orotate and fumaric acid. Surprisingly, hypotaurine, known reported to promotes longevity and stress tolerance in C elegans downregulated in our experiment (Wan et al. 2020). Further studies are needed to understand its specific role in mitochondria and in a human background such as induced pluripotent stem cells. The upregulated metabolites include aspartate, glutamic acid, cystamine and ergocalciferol. Mitochondrial aspartate has been reported to regulate TNF biogenesis and autoimmune tissue inflammation, a key benefit to cells (Wu et al. 2021). With this, we questioned if aspartate may as well confer any neuroprotective benefits in a disease context. We treated our neurons with varying concentrations of aspartate and measured the levels of secreted Aβ peptides, a pathological feature in AD, in the culture media. We observed that aspartate reduced the levels of Aβ 1–42 in a similar magnitude like we see with

HDAC2 knockdown neurons (Fig 2B). This points to a neuroprotective effect of aspartate just like the protective effect in inflammation (Wu et al. 2021).

3.2.3 *HDAC2 knock-down decreases complex I enzymatic activity*

Another route out of the early endosome is via the endocytic recycling complex (ERC) Mitochondrial complex I (CI) catalyzes the transfer of electrons from NADH to ubiquinone while pumping protons across the inner mitochondrial membrane. This electron transfer and proton pumping are essential for generating ATP and maintaining energy production in the cell. Since complex I is the largest subunit of the ETC complexes, we hypothesized that mitochondrial complex I activity will be key to the observed increase in the respiratory profile of neurons upon knockdown of HDAC2. To verify this, we performed an enzymatic activity assay to assess the efficiency of CI. Surprisingly, we observed a decreased CI enzymatic activity in both wild-type and AD cell lines disputing our initial hypothesis of CI as the main driver for the increased OCR in HDAc2 knockdown cells (Fig 3A,3B). This observation had also been reported in previous studies that showed that reduced CI activity or reduced composition of CI and its substrates confers benefits in an AD model and on longevity (McElroy et al. 2022; Dillin et al. 2002; Miwa et al. 2014). One of such studies showed the activation of the AMPK-dependent signaling cascade which led to neuroprotection and a reversal of behavior changes in symptomatic APP/PS1 female mice, a translational model of AD was the resultant effect of the partial inhibition of complex I. AMPK and pAMPK are aimed at restoring energy balance, promoting energy-producing processes, and suppressing energy-consuming pathways in order to maintain cellular and organismal homeostasis. Hence we measured the expression of both AMPK and phosphorylated AMPK to determine if there was a similar mechanism in complex I inhibition by CP2 treatment and HDAC2 knockdown. While AMPK levels showed an upward

trend, there was a remarkedly reduced expression of phospho-AMPK in the HDAC2 KD conditions (Fig 3C). We observed that the CP2-conferred benefit of CI inhibition happens via a different mechanism compared to HDAC2 knockdown. It is worth noting however that using various computational models, it has been shown that a reduced CI activity is neither harmful nor beneficial as long as it doesn't fall below a certain threshold based on the cell type and cell state (McElroy et al. 2022).

3.2.4 *HDAC2 Knock-Down Increases Complex V (CV) Enzymatic Activity*

It has been well studied that reduced or deficient CI activity is able to drive up the activity of other mitochondrial complexes as a compensatory mechanism through the formation of super complexes independent of CI (Alam et al. 2015; Novack et al. 2020; Letts and Sazanov 2017). With this, we focused on understanding the enzymatic activity of complex V (ATP Synthase) and sought to understand HDAC2's knockdown on its function. We observed that knockdown of HDAC2 leads to a remarkable increase in CV activity (Fig 4A,4B). This increase in the enzymatic activity of CV means an enhancement in its ability to catalyze the synthesis of adenosine triphosphate (ATP). Looking back at the RNA-seq results, ATPAF1, a subunit of CV was the most upregulated subunit of the complex five and we confirmed this via mRNA expression (Fig 1F, 4C)

Given that many cellular activities rely on ATP as an immediate energy source, this increase observed in CV activity ensures a more substantial supply of ATP molecules, enabling the neurons to meet their energy requirements more effectively. With this increase, we sought to measure the ATP levels in our HDAC2 KD neurons. Surprisingly, we did see reduced levels of ATP relative to our control in our HDAC2 knockdown cells (Fig 4D). Further treatment by various inhibitors of the mitochondria complexes further showed that as neurons mature, there is

a switch from glycolysis to oxidative phosphorylation. This was evident as neurons treated with 20uM oligomycin (CV inhibitor) had a more reduced ATP expression compared to treatment with 20mM 2DG, an inhibitor of cellular glycolysis. Dual inhibition by both 2DG and oligomycin lead to a near zero in the levels of ATP. Taken together, these data suggests that HDAC2 strongly influences CV activity. However the reduced ATP levels may point to an increased demand by the neurons following production which leads to higher consumption and hence lowered ATP levels when measured. Further experiments at different time points during the maturation process will shed more light on this phenomenon.

3.2.5 *HDAC2 knockdown does not alter mitochondria membrane potential*

The mitochondrial membrane potential ($\Delta\Psi m$) is the electrical potential difference across the inner mitochondrial membrane, which arises from the distribution of ions and the activity of various transport proteins. ATP production relies on this electrochemical gradient generated by the mitochondrial membrane potential (Vasan et al. 2022; Zorova et al. 2018). Realizing the observed changes in complex activity and oxygen consumption, we queried what effect HDAC2 knockdown will have on the mitochondria membrane potential.

To measure the mitochondrial membrane potential, we used a cell permeable cationic dye which accumulates in the mitochondrial matrix based on mitochondrial membrane potential called the tetramethylrhodamine ethyl ester (TMRE) dye, after treating the cells with Rotenone, a complex I inhibitor. We observed that rotenone depolarized the membrane and lead to a reduction in the mitochondrial membrane potential. However in comparing HDAC2 KD neurons treated with rotenone to the control group with rotenone, we didn't see much change (Fig 5A). Similarly, the baseline TMRE levels were unchanged in both control and HDAC2 knockdown neurons . This observation was seen in both wildtype and disease cells. We reported earlier on

about the ability of Endo-B1c over expression to elongate mitochondria. Hence, we measured the mitochondria membrane potential in control cells and cells with Endo-B1c and interestingly, the levels were unchanged – just as we had seen in the HDAC2 treated cells (Fig 5B). This result is indicative of the fact that either the changes observed with the mitochondrial respiration occur independent of the membrane potential or the various genes that influence the state of the membrane potential are not affected upon HDAC2 knockdown or Endo-B1c overexpression.

3.2.6 *HDAC2 Knock-Down does not increase basal levels of lipid peroxidation*

Reactive oxygen species (ROS) are chemically reactive molecules containing oxygen atoms formed as natural byproducts of cellular metabolism. In the final step of oxidative phosphorylation, electrons are transferred along the electron transport chain (ETC), leading to the generation of ATP (Panieri and Santoro 2016; Sies and Jones 2020). However, during this process, some electrons are able to escape from the ETC and react with molecular oxygen, leading to the formation of ROS. Despite the presence of antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase, the highly reactive nature of ROS can cause damage to cellular components such as lipids, proteins, and DNA (de la Lastra Francisco J. Plou and Eduardo Pérez-Lebeña 2022; Lubos, Loscalzo, and Handy 2011; Pigeolet et al. 1990). This process is known as oxidative stress.

With an increased respiration in our HDAC2 knock down neurons, we hypothesized a resulting increase in ROS levels. However due to various challenges in measuring ROS levels, we used a downstream readout of ROS known as lipid peroxidation. Lipid peroxidation occurs when reactive oxygen species (ROS) attack and damage the lipids present in cell membranes leading to the destruction of cellular structures and components. The process of lipid peroxidation forms reactive aldehydes such as malondialdehyde (MDA) and 4-hydroxynonenal

(4- HNE) as natural bi-products (de la Lastra Francisco J. Plou and Eduardo Pérez-Lebeña 2022; Ayala, Muñoz, and Argüelles 2014). We used MDA as a marker of lipid peroxidation in our assay. Surprisingly, despite the increased OCR on the seahorse, there was no change in the levels of basal lipid peroxidation (Fig 6A, 6B). This could mean that the coupling process is more intact upon HDAC2 KD such that there is little to no leakage in our cells. This further supports our initial reports when HDAC2 knockdown confers a neuroprotective ability on neurons (Frankowski et al. 2021). With HDAC2 knockdown, we do see an increase in respiration but a controlled level or no increase in ROS levels.

3.2.7 *HDAC2 knockdown sensitizes neurons to DNA damage by Etoposide*

Maintaining the integrity of the genome is crucial for normal cellular functions and gene expression. Nevertheless, all cells in the body face a constant risk of DNA damage due to factors such as exposure to oxidative stress, genotoxic agents, radiation, and normal cellular processes (Harper and Elledge 2007; Jackson and Bartek 2009). To combat this, cells have developed intricate systems known as the DNA-damage response (DDR), which encompass a range of mechanisms to identify, signal, and repair different forms of DNA damage (Mahaney, Meek, and Lees-Miller 2009; Huertas 2010). This repair mechanism is meant to curtail the detrimental effect of DNA damage and stop their role in the pathogenesis of various diseases such as cancers and neurodegenerative diseases (Alhmoud et al. 2020; Helleday et al. 2008; Pessina et al. 2021; Konopka and Atkin 2022).

Alzheimer’s Disease is no different as increased nuclear and mitochondria DNA damage by oxygen-derived radicals is increased in both early and late onset AD and support the concept that the brain is under stress in such conditions (Bradley-Whitman et al. 2014; Gabbita, Lovell, and Markesbery 1998; J. Wang, Markesbery, and Lovell 2006). Hence we sought to understand

if HDAC2 knockdown may alleviate the occurrence of DNA damage and enhance repair mechanism. We used etoposide DNA damage in transduced cell sand measured the levels of phosphorylated histone H2AX, γH2AX, an early marker of DNA double strand breaks. The cellular DNA damage induced by etoposide commonly results in the formation of single-strand and double-strand breaks, which are widely recognized as the main factors leading to cell death (Montecucco and Biamonti 2007). Etoposide stabilizes the cleavable complex formed between topoisomerase II and DNA, causing these breaks in the DNA strands to occur (Dołega 1998; Walles, Zhou, and Liliemark 1996). We observed an increasing γH2AX levels in cells for 1,3,6, and 12 hours after cells were left to recover following 6hrs of etoposide treatment (Fig 6C). The control cells in contrast repaired the DSBs after the episode treatment was washed off. This observation defies the neuroprotective hypothesis of HDAC2 in regard to DNA damage and supports previously reported work that HDAC inhibition leads to genomic instability (Eot-Houllier et al. 2009). Additionally, it has been reported that the histone hyperacetylation induced by HDAC inhibition causes structural alterations in chromatin, which may expose portions of DNA that are normally protected by heterochromatin to DNA-damaging agents. These findings provide an understanding, in part, of how HDAC2 may function in some instances of neuroprotection but not in all situations.

3.2.8 *MBF-015 provides a promising therapeutic candidate for AD and other related synaptopathies*

Reports in literature have shown HDAC2 as a promising therapeutic target for Alzheimer's Disease due to its link to Aβ overproduction and aggregation, strong correlation with phosphorylated tau and suppression of synaptic genes in various model of AD (Panikker et al. 2018; Nakatsuka et al. 2021; D. Liu et al. 2017; Frankowski et al. 2021; Song et al. 2016).

Hence, inhibition of the HDAC2 isoform has been linked with synapse regrowth, immune suppression and expression of genes relevant for synaptic function. However, its role as a broad epigenetic factor and similarity to other HDACs has made it challenging to target for therapy. Most of the HDAC inhibitors that treat AD models are poorly selective and often cause some undesirable side effects (Yang et al. 2017). Therefore, comprehending the position of individual HDAC in AD pathogenesis is critical to the development of more selective HDAC inhibitors. Medibiofarma, a biotech company in Spain has identified MBF-015 as a potent CoREST/HDAC1/2 inhibitor for the treatment of synaptopathies. MBF-015 is a small molecule which freely crosses the blood-brain barrier and penetrates the brain. Proprietary data from Medibiofarma demonstrates significant induction of synaptic regrowth in animal models and a strong PK-PD relation in the EAE immunosuppression model. Having also shown a reduction in the levels of secreted amyloid beta and an increase in various synaptic genes upon HDAC2 knockdown using lentivirus system, we partnered with the team to test this drug candidate in our hiPSC model. Similarly, we confirmed that MBF-015 is able to reduce amyloid beta levels at 24hrs and over a one-week time point (Fig 7B). Also, we saw a significant increase in the expression level of synaptic genes and genes linked to neuronal maturity (Fig 7A). Taken together, MBF-015 is a potent inhibitor of HDAC2 and we are excited about its potential for therapy as it progresses through clinical development.

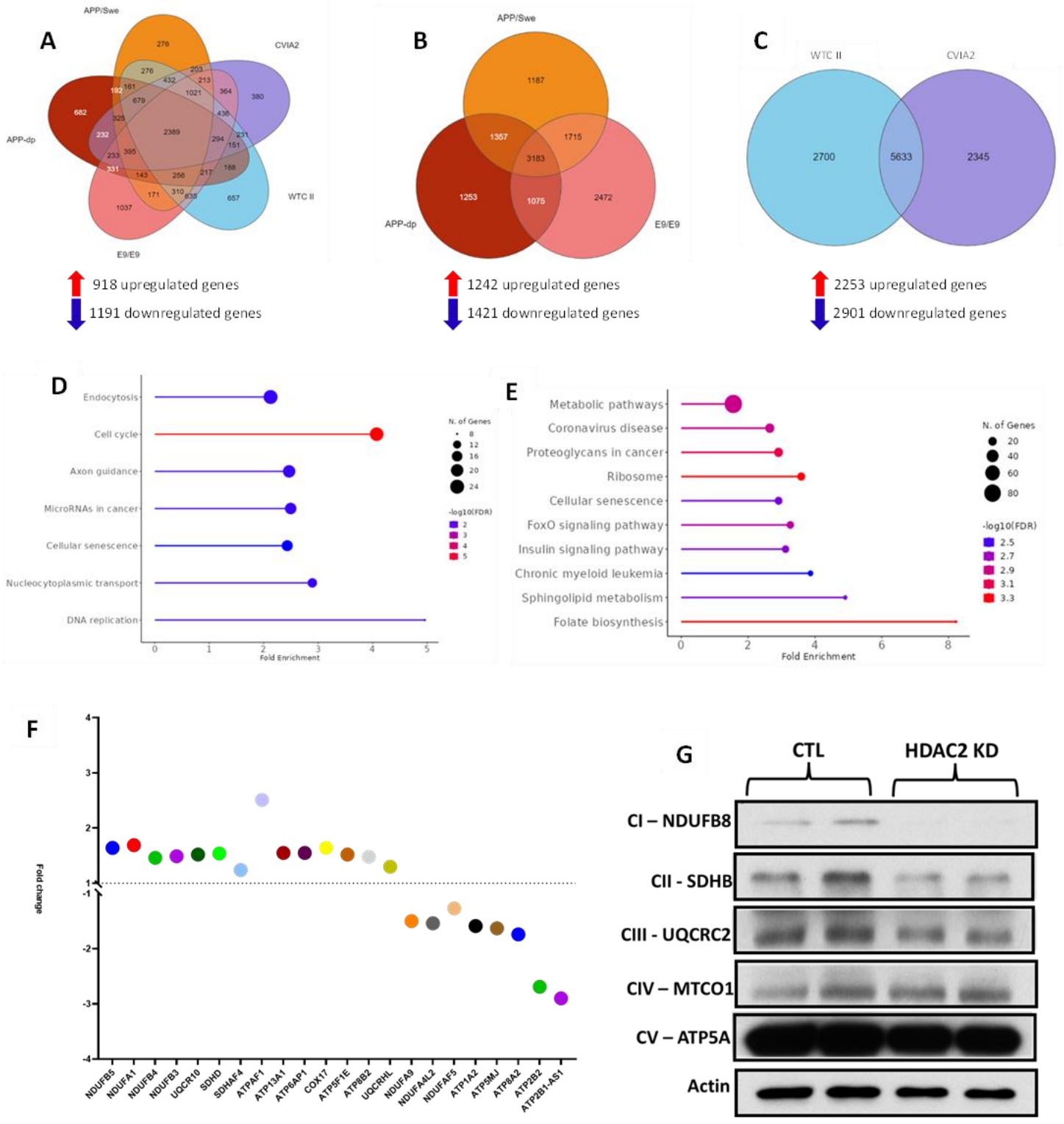

Figure 3.1. **HDAC2 knockdown induces transcriptomic changes in hiPSC-derived neurons**. The surprising observation of more downregulated genes across all cell lines (**A**), in

AD cell lines (**B**) and in wild type cell lines (**C**) upon HDAC2 knockdown. Data was analyzed using Partek software. Shown here are the top ten significantly downregulated (**D**) and upregulated pathways (**E**) upon HDAC2 knockdown using the String GO database. (**F**) Probing experimental result for changes in various mitochondrial genes upon HDAC2 knockdown. (**E**) Confirmation by western blot analysis of selected subunits of the mitochondrial complexes.

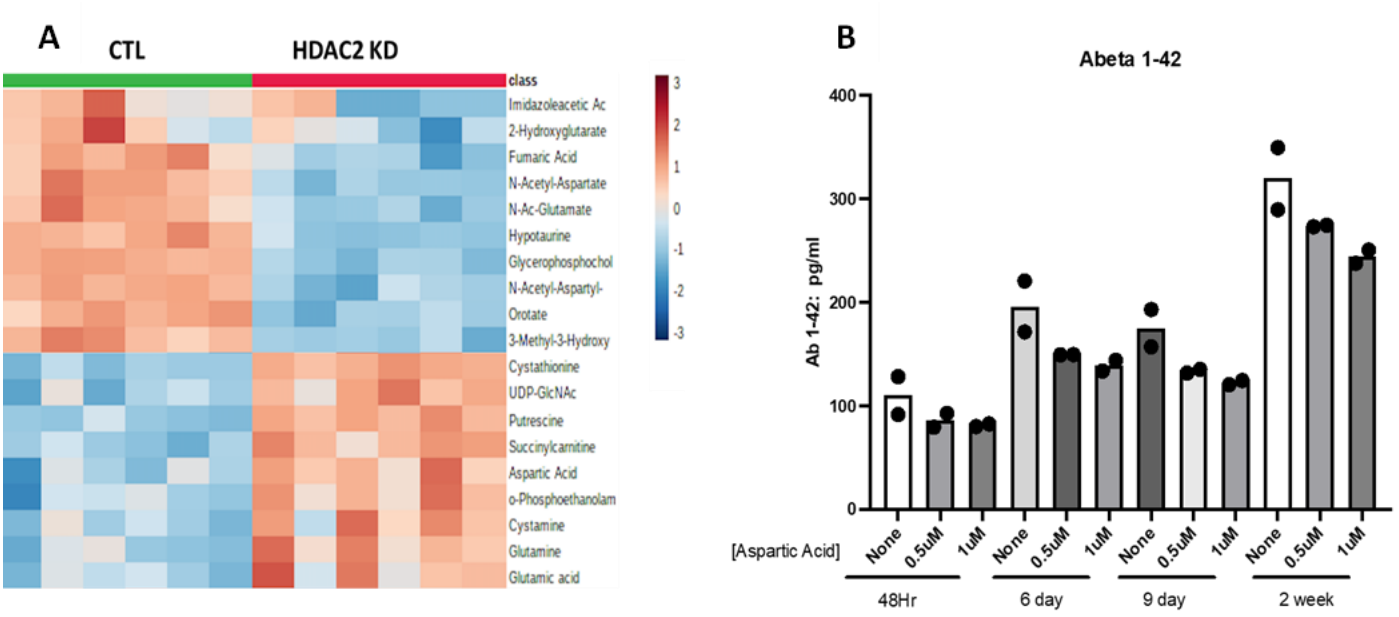

Figure 3.2. **HDAC2 knockdown induces metabolomic changes in hiPSC-derived neurons**.

(A) Heatmap showing significant metabolite expression differences between control and HDAC2 knockdown neurons.

(B) Treatment of neurons with varying concentration of aspartate over different timepoints leads to a reduction in amyloid beta peptide secreted in the culture media. With amyloid beta as a key pathological feature of AD, aspartate, an upregulated metabolite, ability to reduce its levels reveals a neuroprotective effect of aspartate.

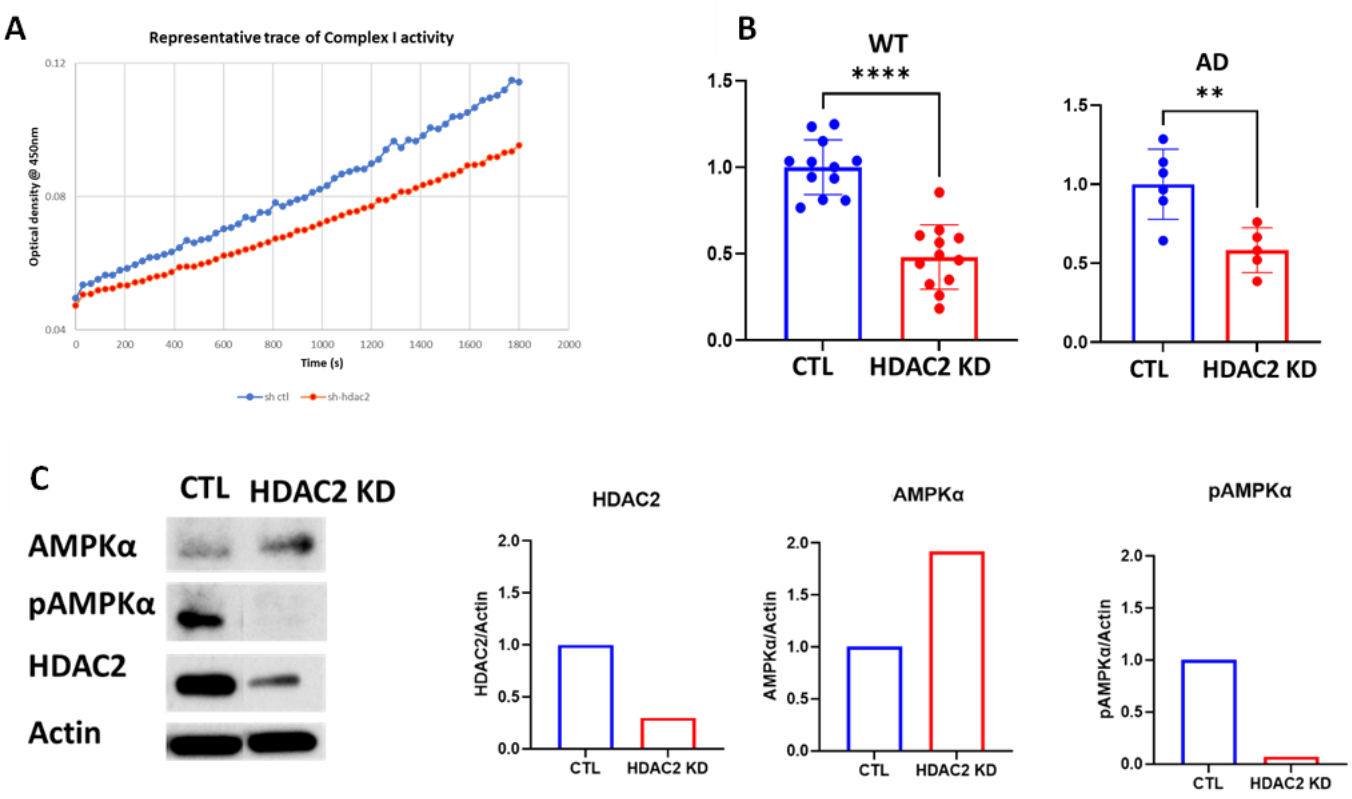

Figure 3.3. **HDAC2 knockdown reduces complex I activity in hiPSC-derived neurons independent of the pAMPKα pathway.**

(A) Representative traces of complex I enzymatic activity over time in wild type and HDAC2 knockdown neurons.

(B) Quantification of activity rate shows roughly 50% reduction in complex I activity in both wild type and AD cell lines.

(C) Western blot analysis to measure pAMPKα levels as a potential explanation for the reduced complex I enzymatic activity assay. pAMPKα levels are drastically reduced when HDAC2 is knocked down. Meanwhile total AMPK levels are elevated. Phosphorylation of AMPK is needed for its function. Therefore with reduced pAMPKα, it suggests the reduced complex I activity is independent of the pAMPKα pathway.

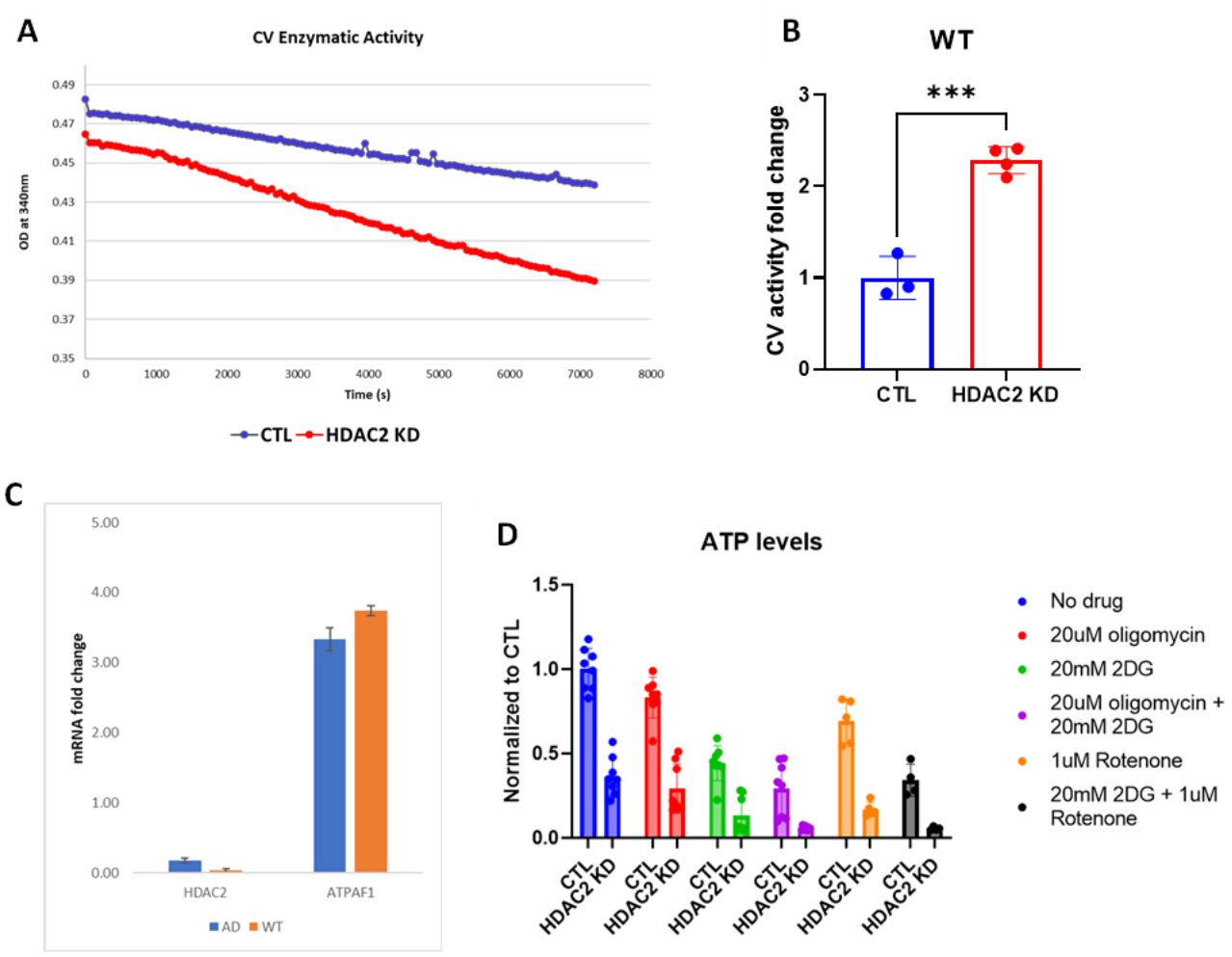

Figure 3.4. **Complex V enzymatic activity increases upon HDAC2 knockdown in hiPSC-derived neurons**. (A) Representative traces of complex V enzymatic activity over time in wild type and HDAC2 knockdown neurons. (B). Quantification of complex V activity rate shows a significant increase in wild type neurons. (C) HDAC2 knockdown induces ATPAF1 mRNA expression in both wild type and AD cell line. (D) Assessment of ATP levels along with inhibitors of the mitochondrial complexes.

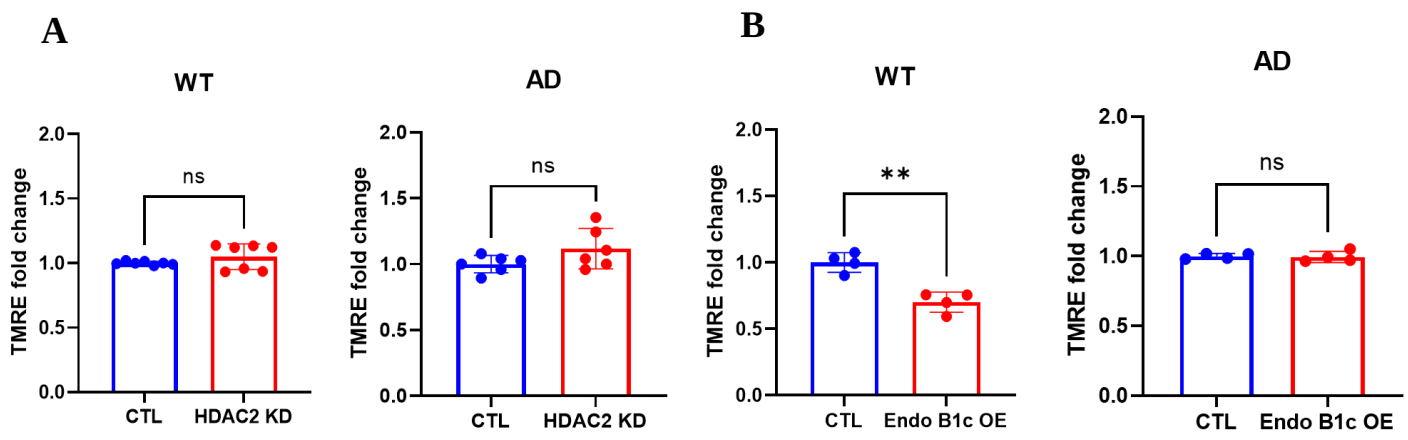

Figure 3.5. **Mitochondria membrane potential remain unchanged in both wild type and AD lines upon knockdown of HDAC2 or Endophilin B1c overexpression.** (A) Knock-down of HDAC2 doesn't alter mitochondrial membrane potential in both wild type and AD cell lines. N=6, two independent neuronal differentiations. (B) Quantification of mitochondrial membrane potential in wild type and AD neurons with overexpression of Endo-B1c. (N = 4)

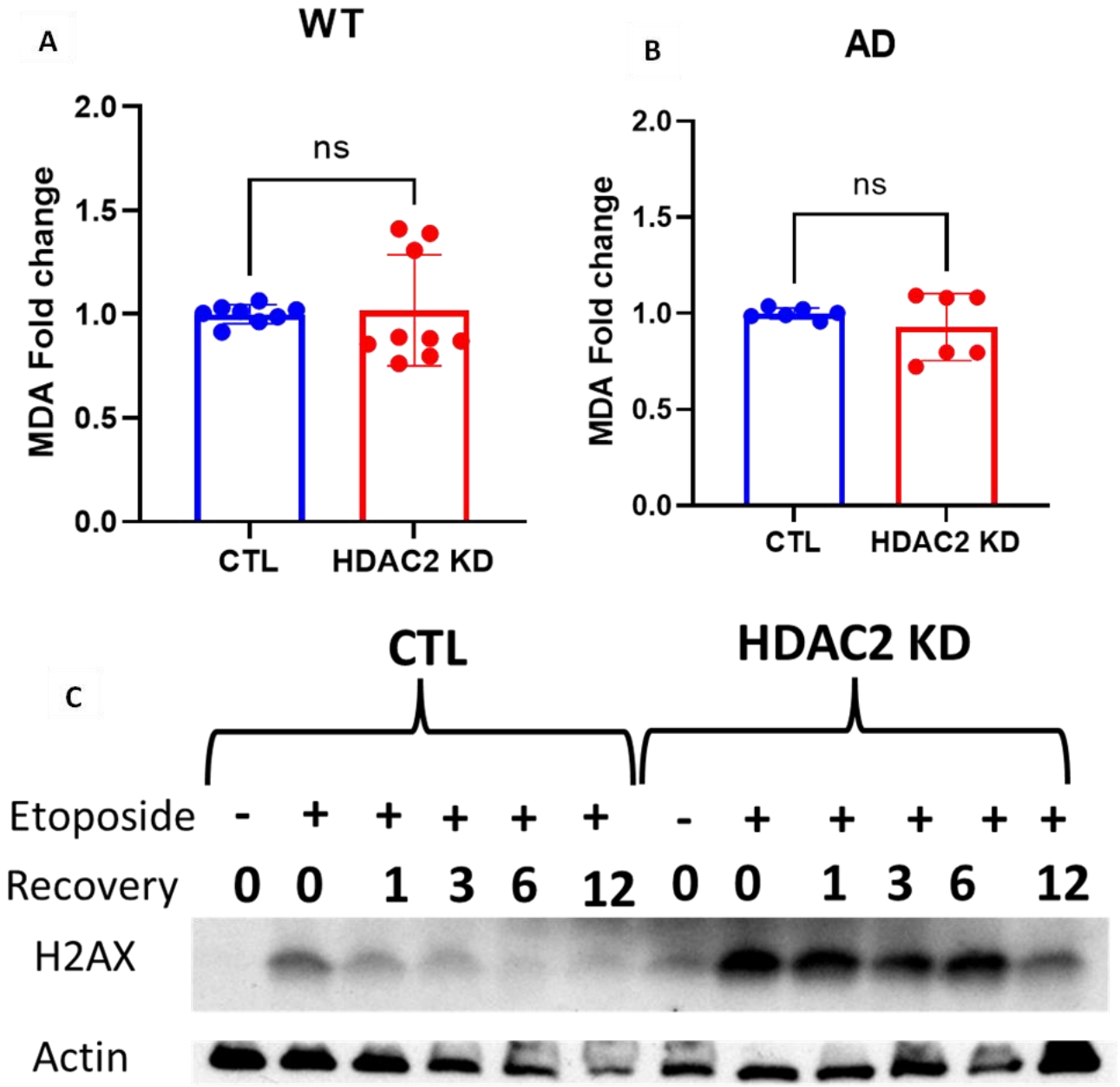

Figure 3.6. **HDAC2 knockdown does not alter lipid peroxidation levels in hiPSC-derived neurons but sensitize neurons to DNA damage induced by etoposide**. Basal levels of lipid peroxidation measured by MDA levels remain unchanged in both wild type (A) and AD cell lines (B). (C) Treatment of cells with 5 µM etoposide for 6hrs induces DNA-damage in hiPSC-derived neurons. After etoposide was removed, cells were left to recover for 1,3,6 and 12 hrs, and the extent of DNA damage was accessed by WB.

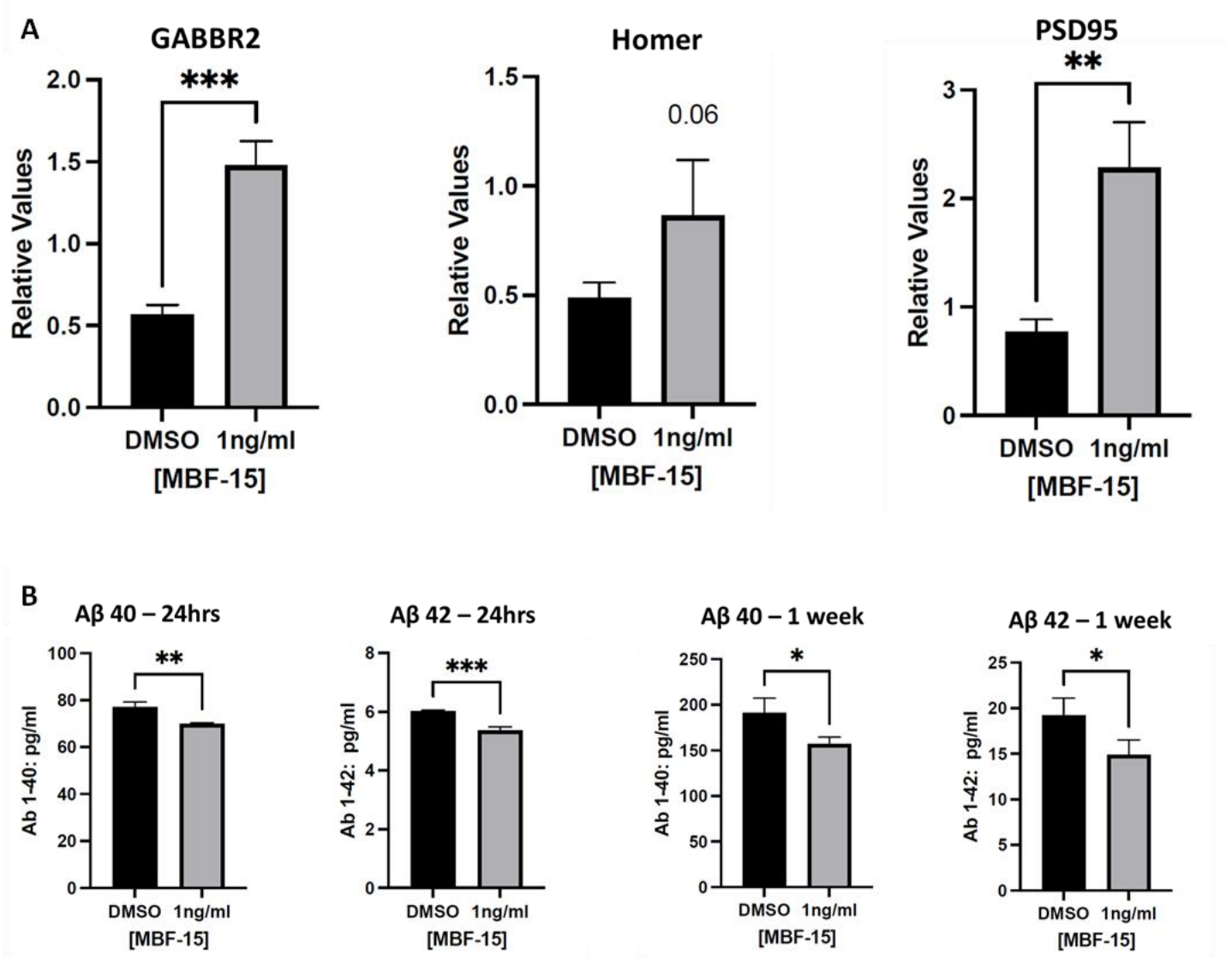

Figure 3.7. **MBF-15 is a promising therapeutic agent for AD**. **(A)** Treatment of neurons with an HDAC2 specific inhibitor, MBF-15, increases mRNA of GABBR2 and PSD95. **(B)** HDAC2 inhibition by MBF-15 reduces Aβ peptides in hiPSC-Ns. Aβ peptides (1–40 and 1–42) secreted in the media from wild type neurons in both 24hrs and 1 week time point.

3.3 DISCUSSION

Through a complex set of chemical reactions, mitochondria breaks down food into energy in the form of adenosine triphosphate (ATP) to drive cellular processes in the body. The rate of metabolism varies from person to person and is influenced by several factors, including age, gender and genetic differences (Pileggi et al. 2022; Heilbronn et al. 2007). From an epigenetic standpoint, a lot of work has focused on the sirtuin family of deacetylases and their role in energy metabolism modulation among others (Chang and Guarente 2014; Haigis and Sinclair 2010; Pfluger et al. 2008). However, recent work from our lab has shown the role of an epigenetic factor, histone deacetylase 2 (HDAC2) on mitochondria gene expression and subsequent increase in respiratory profile via the seahorse assay (Frankowski et al. 2021). We therefore sought to understand the underlying mechanism driving the increase in respiration via the seahorse.

Taking a more global approach, we performed an RNA-seq experiment to understand the transcriptomic changes that occur upon HDAC2 knockdown. The differentially expressed genes and enriched pathways identified in this study contribute to our understanding of HDAC2's regulatory role in gene expression and metabolism. ATPAF1, also known as ATP synthase mitochondrial F1 complex assembly factor 1 was the most upregulated gene in terms of mitochondrial complexes. ATPAF1 The ATPAF1 protein is involved in the assembly of the F1 component of ATP synthase, which is responsible for catalyzing the synthesis of ATP (Zhou et al. 2021). Mutations or dysregulation in the ATPAF1 gene can disrupt the assembly or function of ATP synthase, leading to mitochondrial dysfunction and diseases such as Leigh syndrome and mitochondrial encephalomyopathy (Zhou et al. 2021; Ghezzi and Zeviani 2018). Correlating this to ATP synthase (complex V) activity, we also observed a significant increase in ATP

synthase enzymatic activity. This means that HDAC2 influences complex V and drives up its enzymatic activity and subsequently increases the oxygen consumption rate and respiration in general. Interestingly, we also observed a significant number of downregulated genes especially for complex I. A positive feedback loop system may be responsible for this observation.

Aside the enzymatic activity of the various complexes, the membrane potential was accessed for the increased metabolism of the cell as it provides the driving force for ATP synthesis in mitochondria. Low mitochondria membrane impedes respiration in skeletal muscle mitochondria by a mechanism involving oxaloacetate (OAA) inhibition of succinate dehydrogenase (SDH) (Bai et al. 2016; Fink et al. 2018). On the other hand, the higher the membrane potential, the higher the energy capacity of the inner mitochondrial membrane and the potentially higher the synthesis of ATP (Zorova et al. 2018). Optimal balance is critical for survival and effective functioning of the mitochondria. Moreover when briefly depolarized in a process known as flickering, the mitochondria can release the accumulated unwanted substances, such as cations (Dmitry B. Zorov, Juhaszova, and Sollott 2006; D. B. Zorov et al. 2000). Longer periods of depolarization leading to reduced membrane potential destroys the mitochondria, a phenomenon seen in aging as the integrity of the mitochondria membrane potential is destroyed (D. B. Zorov, Kinnally, and Tedeschi 1992; Jin et al. 2010). In our model, we observed an unchanged mitochondrial membrane potential and hence cannot attribute it as a direct benefit or a disadvantage upon HDAC2 knockdown.

The mitochondrial free radical theory of aging proposes that the progressive mitochondrial dysfunction that occurs with aging results in increased production of ROS, which in turn causes further mitochondrial deterioration and global cellular damage (López-Otín et al. 2013; Dmitry B. Zorov, Juhaszova, and Sollott 2006; Lagouge and Larsson 2013). Also, ROS is

known as a by-product of mitochondria respiration. With aging as the main risk factor in AD, we sought to understand if HDAC2 knockdown conferred any neuroprotective effect of the production of ROS. Using MDA, a product of lipid peroxidation as a readout, we observed no changes in the levels of lipid peroxidation between the control cells and HDAC2 knockdown cells in both disease and wild type cell lines. Also, normal metabolic processes within cells can lead to DNA damage. The DNA of the cell contains genetic information for making various genes. DNA damage refers to any alteration or disruption in the structure of DNA molecules. Here, we analyzed the effect of HDAC2 on DNA damage. We realized that HDAC2 knockdown sensitizes neurons to DNA damage induced by etoposide. This finding was in line with previous reports that showed that during chromatin remodeling, DNA damage are remarkedly high phenotypes that reflect defective DSB repair, particularly by nonhomologous end-joining (NHEJ) (Price and D'Andrea 2013; B. Liu, Yip, and Zhou 2012). Also, HDAC1 and HDAC2 are two participants in the DNA-damage response. Acetylation of histone H3 Lys56 (H3K56) is regulated by HDAC1 and HDAC2 and HDAC1 and HDAC2 are rapidly recruited to DNA-damage sites to promote hypoacetylation of H3K56 (Miller et al. 2010).

Current treatments for AD disease including cholinesterase inhibitors and memantine have limited effect for a short duration (Huang, Chao, and Hu 2020; Reisberg et al. 2003). Therefore there is a pressing need to develop better therapies to relieve the cognitive impairments of the disease and improve the quality of life. MBF-15 so far has shown potency in upregulating synaptic genes all while decreasing amyloid beta levels. It is yet to be seen whether it will help against tau and other comorbidities reported in Alzheimer's.

Understanding HDAC2's functions and the downstream effects of HDAC2 dysregulation can provide insights into the epigenetic regulation of gene expression and potentially identify

new targets for therapeutic interventions. These findings provide an understanding, in part, of the potential of epigenetic regulation on a cellular process – metabolism and how HDAC2 could be a potential target for Alzheimer's.

3.4 METHODS

3.4.1 *hiPSC neuronal differentiation*

hiPSCs were differentiated to neurons using dual-SMAD inhibition (Chambers et al. 2009). Briefly, hiPSCs were plated on Matrigel coated 6-well plates at a density of 3.5 million cells per well and fed with Basal Neural Maintenance Media (1:1 DMEM/F12 + glutamine media/neurobasal media, 0.5% N2 supplement, 1% B27 supplement, 0.5% GlutaMax, 0.5% insulin-transferrin-selenium, 0.5% NEAA, 0.2% β-mercaptoethanol; Gibco, Waltham, MA) + 10 mM SB-431542 + 0.5 mM LDN-193189 (Biogems, Westlake Village, CA). Cells were fed daily for 7 days. On day 8, cells were incubated with Versene, gently dissociated using cell scrapers, and passaged at a ratio of 1:3. On day 9, the media was switched to Basal Neural Maintenance Media and fed daily. On day 13, the media were switched to Basal Neural Maintenance Media with 20 ng/ml FGF (R&D Systems, Minneapolis, MN) and fed daily. On day 16, cells were passaged again at a ratio of 1:3. Cells were fed until approximately day 23. At this time, cells were FACS sorted to obtain the CD184/CD24-positive and CD44/CD271-negative neural precursor cell (NPC) population. Following sorting, NPCs were expanded for neural differentiation. For cortical neuronal differentiation, NPCs were plated out in 10 cm cell culture dishes at a density of 6 million cells/10 cm plate. After 24 h, cells were switched to Neural Differentiation media (DMEM-F12 + glutamine, 0.5% N2 supplement, 1% B27 supplement, 0.5% GlutaMax) + 0.02 μg/ml brain-derived neurotrophic factor (PeproTech, Rocky Hill, NJ) + 0.02 μg/ml glial-cell-derived neurotrophic factor (PeproTech) + 0.5 mM dbcAMP (Sigma

Aldrich, St Louis, MO). Media was refreshed twice a week for 3 weeks. After 3 weeks, neurons were selected for CD184/CD44/CD271-negative population by MACS sorting and plated for experiments

3.4.2 *Neuron Purification*

After 3 weeks of differentiation, neurons were dissociated with accutase and resuspended in Magnet Activated Cell Sorting (MACS) buffer (PBS + 0.5% bovine serum albumin [Sigma Aldrich, St Louis, MO] + 2 mM ethylenediaminetetraacetic acid [Thermo Fisher Scientific, Waltham, MA]). Cells were incubated with PE-conjugated mouse anti-Human CD44 and mouse anti-Human CD184 antibodies (BD Biosciences, San Jose, CA) at a concentration of 10 µl/10 million cells. Then, cells were washed with MACS buffer and incubated with anti-PE magnetic beads (BD Biosciences, San Jose, CA) at a concentration of 50 µl/10 million cells. Bead-antibody complexes were pulled down using a rare-earth magnet, supernatants were selected, washed, and plated at an appropriate density.

3.4.3 *Measurement of complex I enzymatic activity*

Measurements of complex I activity was performed in wild type cell, CVIA2 and the APP/Swe Alzheimer's cell line using the manufacturer's protocol (Abcam ab109721). Cells were harvested and equal lysate concentration according to the protein amount measured by a BSA assay from both control and HDAC2 KD cells were loaded onto the assay plate for 3 hrs. After three rounds of washes, an assay solution was added and the optical density at 450nm was measured in kinetic mode for 1 hour at 30 sec intervals. The complex activity is indicated as the change in absorbance per minute (mOD/min).

3.4.4 *Measurement of complex V (ATP Synthase) enzymatic activity*

The activity of complex V (ATPase) was measured with Abcam (ab109714) kit. The ATP synthase complex was immunocaptured within the wells in the microplate. To measure its activity, the enzymatic hydrolysis of ATP is coupled to oxidation of NADH and recorded by measuring the decrease in absorbance at 340 nm on a microplate reader. The activity of CV is expressed as the decrease in absorbance at 340 nm.

3.4.5 *Mitochondria membrane potential*

Purified neurons were seeded at a density of 5x106 cells/ml of media in a 24 well plate. The neurons were transduced with HDAC2 and treated with 100 nM TMRE (Cayman chemical #701310) for 30mins before reading the fluorescence via flow cytometry.

3.4.6 *Amyloid beta measurement*

Aβ peptides were measured as previously described (Frankowski et al. 2021). Briefly, purified neurons were plated in 96well plates at a density of 200,000 cells per well. Cell lysates and media were harvested from triplicate wells. To measure Ab peptides, an Ab Triplex ELISA plate (Meso Scale Discovery #151200E-2) was used to assess media.

3.4.7 *RNA-sequencing*

RNA was collected from 3 separate differentiations including a combination of two WT lines and three AD lines. Each sample includes 3 technical replicates. RNA was collected from 3 million purified neurons for each sample. The purification of total RNA was completed using the PureLink RNA Mini Kit (Thermo Fisher 12183018A). Assessment of purified RNA was completed using a NanoDrop. Library preparation was completed using the TruSeq Stranded

mRNA kit (Illumina RS-122–2103) per manufacturer instructions. Sequencing was performed on a NovaSeq 6000 instrument. Partek software was used to analyze the results. STRING and Shiny-GO were used to generate the GO-terms

3.4.8 *Western Blot Analysis*

2×10^6 cells were lysed in 200 μL RIPA buffer and protein amount was assessed using BCA assay kit (Thermo Scientific, Waltham, MA, USA). Between 10 and 15 μg of total protein were loaded on to a 4–15% gradient TGX gel (Bio-Rad) and transferred on to a PVDF membrane (Bio-Rad). Following blocking with 5% nonfat dry milk, 0.1% Tween 20 and 0.05% thimerosal, membrane was incubated with the primary antibody diluted in 5% bovine serum albumin, 0.1% Tween 20, 0.05% thimerosal and 0.2% NaN3 overnight at 4 °C, followed by horse radish peroxidase-conjugated secondary antibody (1:2000, GE Healthcare, Chicago IL, USA) diluted in the blocking buffer. Membrane was then developed using Clarity Western ECL substrate (Biorad, Hercules, CA, USA) and exposed to Hyperfilm ECL (GE Healthcare, Chicago IL, USA). Films were digitally scanned, and band intensity was quantitated using ImageJ and normalized against β-actin.

3.4.9 *Antibodies*

The following primary antibodies were used: HDAC2 (Sigma H2663) at 1:5000; Total OXPHOS Rodent WB Antibody Cocktail (ab110413, abcam) at 1:1000; Phospho-AMPKα Thr172 (Cell signaling #2535) at 1:1000, AMPKα D5A2(Cell signaling #5831) at 1:1000; Anti-Histone H2A.X (Millipore Sigma #07-627) and actin (Sigma A5441) at 1:2500

3.4.10 *RNA Purification and qPCR Analysis*

Total RNA was purified from 2 × 105 cells using TRIzol (Life Technologies, Carlsbad, CA, USA) followed by 1st strand cDNA synthetized using the iScript kit (Biorad, Hercules, CA, USA). Between 5 and 10 ng of cDNA were used in a 4 uL reaction using POWEUP SYBR qPCR mix (Life Technologies, Carlsbad, CA, USA). All primer-sets were run in technical triplicates. Expression was calculated using the 2—ΔΔCt method and genes of interest were normalized to RPL27. qPCR primers designed over exon-exon boundaries using Primer-Blast are below:

HDAC2 - TGAGATTCCCAATGAGTTGCCA/TACTGACATCTGGTCAGACA

ATPAF1 – CAGTGCATCGCCAACCAAG/TTAAAGGTCTCCACTAACCCGT

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Chapter 4. CONCLUSION

4.1 INTRODUCTION

Alzheimer's disease (AD) is a progressive and irreversible neurodegenerative disorder that primarily affects the brain, leading to cognitive decline and memory loss (Knopman et al. 2021; Moya-Alvarado et al. 2016). Named after Dr. Alois Alzheimer (Tagarelli et al. 2006), who first described the condition in 1906, it is the most common form of dementia and mainly affects older adults, although early-onset cases can occur. AD's early symptoms often involve mild forgetfulness and difficulty with short-term memory. As it progresses, individuals may experience confusion, disorientation, language problems, and challenges in completing familiar tasks. The buildup of abnormal protein structures in the brain, namely amyloid plaques and tau tangles are the main pathological features of the disease (Muralidar et al. 2020; Brodaty et al. 2011; Eftekharzadeh et al. 2018; Makin 2018). These deposits interfere with the communication between nerve cells, leading to their dysfunction and eventual death. While the exact cause of Alzheimer's remains unknown, certain risk factors have been identified. These include age, family history, genetics (specifically the presence of the APOE ε4 gene), and lifestyle factors such as cardiovascular health, diet, and exercise (López-Otín et al. 2013; Barber 2012).

AD typically progresses through three main stages - early (mild), middle (moderate), and late (severe). Each stage is associated with increasing cognitive and functional impairment. As the disease advances, individuals may struggle with basic activities of daily living, lose their ability to communicate effectively, and may require full-time care and assistance. The diagnosis requires a thorough medical evaluation, including medical history, cognitive tests, and imaging studies to rule out other potential causes of cognitive impairment. Currently, there is no cure for Alzheimer's disease. The available treatment options aim to manage symptoms and may include

medications to slow cognitive decline and improve quality of life (Fink et al. 2018). Most of the failed drug candidates focused on tau and amyloid beta (Huang, Chao, and Hu 2020; VandeVrede, Boxer, and Polydoro 2020). However there is a need to turn to other pathways independent of tau and amyloid beta.

Histone Deacetylase 2 (HDAC2) has emerged as a potential therapeutic target for AD due to its involvement in epigenetic regulation and its impact on gene expression. Such epigenetic mechanisms modify gene activity without altering the primary DNA sequence (Gibney and Nolan 2010). HDAC2, in particular, is an enzyme that plays a crucial role in histone deacetylation, leading to a more compact chromatin structure and repression of gene transcription (Yamakawa et al. 2017; Guan et al. 2009). In the context of AD, several studies have implicated HDAC2 in the formation of amyloid plaques and tau tangles, which are hallmarks of the disease (Liu et al. 2017). Additionally, HDAC2 has been associated with synaptic dysfunction and impaired memory formation, further linking it to AD pathogenesis. HDAC2 inhibition may help reduce tau hyperphosphorylation and prevent tau-related neurotoxicity. However, while HDAC2 inhibition shows promise, there are challenges in developing safe and effective therapies targeting HDAC2. This is because the various HDAC isoforms share commonalities and selectively targeting HDAC2 while avoiding off-target effects has not been super successful (Shukla and Tekwani 2020; Xu et al. 2011).

4.2 KNOCK-DOWN OF HDAC2 IN HUMAN INDUCED PLURIPOTENT STEM CELL DERIVED NEURONS IMPROVES NEURONAL

MITOCHONDRIAL DYNAMICS, NEURONAL MATURATION AND REDUCES AMYLOID BETA PEPTIDES

HDAC2 has been shown to mediate various cellular process which underlie the neurodegenerative pathologies seen in Alzheimer's Disease (Jawerka et al. 2010; Mahady et al. 2019). This makes HDAC2 an attractive therapeutic target. We therefore sought to understand how HDAC2 may affect various cellular processes using human induced pluripotent stem cells. In this study, we demonstrate a progressive decrease in HDAC2 levels during neuronal differentiation, which is accompanied by a natural increase in neuron-specific isoforms of Endophilin-B1 (Endo-B1b/c). Aside from this endogenous relationship between HDAC2 and Endo Endo-B1b/c, we observed a similar pattern using lentivirus system to knockdown HDAC2 in hiPSC-derived cortical neurons. This confirmed that HDAC2 regulates the expression of Endo-B1b/c in human neurons. Additionally, we investigated the roles of HDAC2 and Endo-B1b/c in human neurons using knock-down and overexpression experiments. Our results show that both knock-down of HDAC2 and overexpression of Endo-B1c promote mitochondrial elongation and protect neurons from cytotoxic stress. However, only HDAC2 knock-down affects the expression of genes involved in mitochondrial gene expression and mitochondrial respiration.

Furthermore, we observed that reducing HDAC2 levels in hiPSC-Ns leads to a decrease in the levels of Amyloid beta (Aβ), a cleavage product of the amyloid precursor protein (APP) implicated in neurotoxicity in Alzheimer's disease (AD) (Makin 2018). Our findings provide evidence in a human neuronal cell model for the role of HDAC2 in modulating neuronal synaptic gene expression and implicate several pathways through which HDAC2 regulates mitochondrial dynamics and physiology in hiPSC-Ns. Taken together, these results suggest that HDAC2

inhibition may represent a potential therapeutic strategy for AD and other neurodegenerative disorders.

4.3 CHARACTERIZING THE ROLE OF HDAC2 IN NEURONAL METABOLISM IN HIPSC-DERIVED NEURONS

Mitochondria is often known as the powerhouse of the cell because it play a central role to energy generation in the form of ATP through oxidative phosphorylation and the citric acid cycle (Krebs cycle). HDAC2 on the other hand, might modulate mitochondrial function indirectly through its involvement in regulating the expression of mitochondrial genes (Milstone et al. 2020; Y. Wang et al. 2019; D. B. Wang et al. 2019). In a previous study, we showed that HDAC2 regulates EndophilinB1-b/c levels and also leads to an increase in the oxygen consumption rate in hiPSC-derived neurons (Frankowski et al. 2021). HDAC2 has broadly also been shown to influence the expression of genes involved in glucose metabolism, lipid metabolism, and oxidative stress responses, which ultimately can impact mitochondrial function (King, Patel, and Chandrasekaran 2021; Nguyen et al. 7 2020). Additionally, HDAC2 has been found to interact with certain transcription factors and co-regulators that are known to be involved in mitochondrial biogenesis and function, further supporting its potential role in mitochondrial metabolism. However, the direct mechanism underlying these observations is yet to be elucidated.

In this study, we ran a bulk RNA-seq and a targeted metabolomic assay to identify the key transcriptomic changes and metabolites that change upon regulation of HDAC2. We observed both upregulated and downregulated genes and metabolites across board in both wild type and AD cell lines. Surprisingly, we saw more downregulated hits despite HDAC2's knockdown which generally leads to a more open chromatin state allowing for more gene

transcription. We followed up the observations here with various phenotypic assays to understand the underlying mechanism of neuronal metabolism following HDAC2 down regulation. The enzymatic activity of mitochondrial complex I was significantly reduced while complex V (ATP synthase) activity was significantly increased. ATPAF1, a component of complex V was also among the highly upregulated transcripts from the RNA-seq experiment.

The levels of reactive oxygen species (ROS) are known to increase with increased respiration (Tiwari, Belenghi, and Levine 2002). The effects of this increase could be detrimental to the cells. We measured the levels of ROS through the lipid peroxidation and didn't observe any significant changes in either wild type or AD cell lines. We also measured the mitochondrial membrane potential and didn't observe any difference between the control and HDAC2 knockdown cells as well. Finally, we tested the efficacy of a phase I/II small molecule inhibitor of HDAC2and observed an increased expression of synaptic genes and a decrease in levels of amyloid beta peptides in the culture media. While the exact mechanism of HDAC2 on neuronal metabolism has not been teased out yet, we hope this body of work will shed more light on the contribution of HDAC2 and grant further insights into the epigenetic regulation of neuronal metabolism.

4.4 CONCLUSION

The increased expression of HDAC2 in postmortem brains of AD patients coupled with the repressive attribute of HDACs is an interesting puzzle to study. In AD, synaptic function is perturbed and HAC2 has been found to repress genes necessary for synaptic function. HDAC2 was recently shown to regulate mitochondrial genes and function. In AD, mitochondrial dysfunction is also a major player in the disease pathogenesis. Therefore, understanding the role of HDAC2 in AD progression will help design effective therapies to curb disease progression if

not totally cure AD. Here, we determine that HDAC2 knockdown increased the expression of synaptic genes, increase mitochondrial respiration and reduced Aβ secretion. Additionally, we show that we can rescue the AD defects using a small molecule – MBF-15. Finally, we have shown that HDAC2 regulates mitochondrial complex activity by reducing complex I activity while enhancing complex V activity. However this increased respiration is not driven by the mitochondrial membrane potential and neither is there an increase in the amount of reactive oxygen species measured by lipid peroxidation. Collectively, this work shows that the inhibition of HDAC2 is a valid therapeutic target and may ameliorate mitochondria and synaptic defects seen in AD.

For future directions, an understanding of all the mitochondrial complexes and the resulting effect of HDAC2 KD on their enzymatic activities will provide a complete picture of how HDAC2 regulates mitochondrial function. Additionally, despite the increase in the levels of synaptic genes, it will be interesting to determine the formation of synapses and firing efficiency upon HDAC2 KD. Not much work has been done on tau, another pathologic hallmark of AD, in regards to HDAC2's ability to regulate its activity. Using hiPSCs to query the effect of epigenetic modifications on tau will further help understand HDAC2's role in tauopathies.

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