Basal Forebrain Cholinergic Neurons in Down Syndrome

by

Jose L. Martinez

A dissertation submitted in partial fulfillment of

the requirements for the degree of

Doctor of Philosophy

(Cellular and Molecular Biology)

at the

UNIVERSITY OF WISCONSIN-MADISON

2024

Date of final oral examination: 01/04/2024

The dissertation is approved by the following members of the Final Examination Committee:

Anita Bhattacharyya, Professor, Cell and Regenerative Biology
Barbara B. Bendlin, Professor, Geriatrics and Gerontology
Bradley T. Christian, Professor, Medical Physics and Psychiatry
Darcie L. Moore, Associate Professor, Neuroscience
Xinyu Zhao, Professor, Neuroscience

DEDICATION

I dedicate this thesis to my grandparents, Maria Luisa Gomez, and Jesus Martinez, who influenced countless people in their time here on earth. Although you didn't have the chance to see this adventure, thank you for believing in me and motivating me to pursue what I once thought was impossible. I love you and miss you more than words can say.

ACKNOWLEDGMENTS

The completion of this Ph.D. has been a challenging and rewarding experience that I couldn't have done without the help and support of many. Taking on this journey away from home, starting over in a new state, and not being able to see the major milestones in my family's lives has been a struggle. However, over the years I have met wonderful people who have made this journey a memorable one. I would like to take this opportunity to acknowledge them:

To my Dr. Anita Bhattacharyya and the Bhattacharyya Lab:

Thank you for taking a risk with me as your first graduate student. Even though I had no experience in stem cell culture, neuroscience, and developmental biology, you provided an environment in which I could grow and develop into the scientist I am today. Thank you to past and current Bhattacharyya Lab members for making our lab a cool lab and making my time in graduate school a pleasant one. You are all fantastic scientists and more importantly, you are great people.

To my Family:

Thank you for being an inspiration to me since day one. Maria Luisa Gomez, Jesus Martinez, Gloria Fuentes, Jose Luis Martinez Sr., Aurora Carbajal, Edith Guadalupe Martinez-Fuentes, and David Martinez, you all have gone through challenging circumstances in life and yet have persevered. Your experiences, encouraging words, and playful sibling rivalry, have been a big motivator for me to not give up throughout this journey. Alyssa Dooley, Chris Dooley, Holly

Dooley, and Brendan Dooley, thank you for making Wisconsin feel like home. I love you all tremendously.

To my Friends:

Thank you to all of you for your support and for making this challenging journey a more bearable one. Drake Ortega and Dr. Karen Ruiz, you both have been there with me since our times as banana slugs at UC Santa Cruz. You both encouraged me to pursue goals I didn't think I could achieve. Carissa and Nicole, the two of you are some of the best lab mates and friends. Thank you for always being available to bounce ideas off you, grab food, and join whatever random rec league sport I signed up for. David, Jaime, Jesus, and Matt, those spontaneous trips to places like pajarete, Bucks basketball games, or USFL Michigan Panthers football games. All key adventures allowed me to maintain a work-life balance that I didn't know I needed at the time. Erin, Austin B., Brett, Jamie, Annalise, Austin R., Cassie, and Tristan, thank you guys for providing laughs, good conversations, and fun times on the volleyball court and kickball fields. I know that if we had played together one more year the championship would be ours. Sharon and Luis, the two of you have ben key friends in getting through our CMB Ph.D. program together. Thank you for the best brunch in town and endless laughs. Hecmarie and Bo, you both are inspirations to me. To all of you and those who I may have missed, I am honored to call you friends and proud of each one of you. I look forward to continuing these friendships and seeing all the amazing things you will all accomplish in life.

To my Mentors:

Never have I believed the saying "it takes a village" more than I do as I come to the end of this Ph.D. journey. Thank you for your invaluable advice, support, and willingness to make time to speak with me. You all have had a unique impact in my life, both personally and professionally. I am thankful for your leadership. *Dr. Pradip Mascharak, Dr. Margarita Gonsalez, Yulianna Ortega, Nancy Cox-Konopelski, Dr. Tepring Piquado, Dr. Ben Allen, Dr. Kate F. Barald, Dr. Asim Beg, Georgina Nicholl, Michelle DiMondo, and Abbey Thompson.*

To my Wife:

No words can ever describe how lucky I am to call you, my wife. Alyssa, you have been a source of inspiration since the first time I met you. Your intelligence, relentlessness to achieve your goals, and kind heart have all made me a better person and scientist. Thank you for supporting me and not letting me quit. You've been supportive in many ways, but more so over the last year living in different states. I am grateful that you not only showed empathy and care via countless phone calls, but also motivated me through your work ethic. I look forward to our new adventure together with our three pets: Callie, Penny, and Lumi. I love you!

ABSTRACT

Down syndrome (DS), or trisomy 21 (T21), is both a complex neurodevelopmental and neurodegenerative disorder. DS is characterized by altered neuronal cortical development resulting in intellectual impairment at birth and Alzheimer's Disease (AD) pathology in middle age (Coyle et al., 1986; Scott et al., 1983). It is estimated that more than 400,000 individuals are living with DS in the U.S. at any given time. As individuals with DS age, these cognitive functions decline as they develop AD pathology. A critical feature that links cognitive impairment and neurodegeneration in trisomy 21 is the population of neurons that are susceptible in both processes: basal forebrain cholinergic neurons (BFCN). Degeneration or impairment of BFCNs leads to memory loss, decreased spatial recognition, and disturbance in language. Degeneration of this population has also been well-documented in a diverse range of human neurocognitive disorders, including DS and AD. However, key molecular pathways involved in BFCN degeneration leading to cognitive, memory, and learning deficits aren't well understood.

This thesis explores the unique differences of BFCNs derived from both isogenic euploid and DS human-derived induced pluripotent stem cells (iPSCs) to better understand the molecular mechanisms that underlie the susceptibility of this subset of neurons. An optimized protocol to differentiate hPSC to BFCNs is presented in Chapter Two, resulting in an efficiency of ~60%. Chapter Three T21 BFCN scRNAseq analysis revealed genes involved in apoptosis, inflammation, mitochondrial dysfunction, and proteinopathies. Additional analysis revealed no statistical differences in apoptosis or tau phosphorylation, but did reveal a significant increase of amyloid-β. Results provide foundational data for identifying therapeutic targets for BFCNs in DS, as well as have an impact on our overall understanding of other neurodegenerative diseases such as AD.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

Term	Abbreviation
Alzheimer's Biomarker Consortium – Down syndrome	ABC-DS
Alzheimer's Disease	AD
Alzheimer's Disease Neuroimaging Initiative	ADNI
Amyloid Precursor Protein	APP
Basal Forebrain Cholinergic Neurons	BFCNs
Bone Morphogenetic Protein 9	BMP-9
Brain Derived Neurotrophic Factors	BDNF
Central Nervous System	CNS
Cerebrospinal Fluid	CSF
Choline Acetyltransferase	ChAT
Down syndrome	DS
Down syndrome related Alzheimer's Disease	DS-AD
Down-Alzheimer Barcelona Neuroimaging	DABNI
Duplication of APP	Dup-APP
Embryoid Bodies	EB
Histamine H1 receptor	H1R
Human Chromosome 21	Has 21
Human Embryonic Stem Cell	hESC
Human Pluripotent Stem Cells	hPSCs
induced Pluripotent Stem Cells	iPSC
Laser Capture Microdissection	LCM
Lateral Ganglionic Eminences	LGE
Magnetic Resonance Imaging	MRI
Medial Ganglionic Eminences	MGE
Murine	mmu
Neural Progenitor Cells	NPCs
Neurofibrillary Tangles	NTFs
Neurospheres	NS
Neurotrophin Receptor	NTR
Nucleus basalis of Meynert	NbM
Parkinson's disease	PD
Positron Emission Tomography	PET
Sant Pau Initiative on Neurodegeneration	SPIN
Sonic Hedgehog	SHH
Trisomy 21	T21
Unites States	U.S.

CHAPTER ONE: BASAL FOREBRAIN CHOLINERGIC NEURONS: LINKING DOWN SYNDROME AND ALZHEIMER'S DISEASE

Jose L. Martinez[1,5], Matthew Zammit[2,5] Nicole West[1,5], Bradley T. Christian[2,4,5], Anita Bhattacharyya[3,5*]

[1]Cellular and Molecular Biology Graduate Program, University of Wisconsin, Madison, Wisconsin

[2]Department of Medical Physics, School of Medicine and Public Health, University of Wisconsin, Madison, Wisconsin

[3]Department of Cellular and Regenerative Biology, School of Medicine and Public Health, University of Wisconsin, Madison, Wisconsin

[4]Department of Psychiatry, School of Medicine and Public Health, University of Wisconsin, Madison, Wisconsin

[5]Waisman Center, University of Wisconsin, Madison, Wisconsin

*** Correspondence:**

Anita Bhattacharyya, Ph.D.

bhattacharyy@waisman.wisc.edu

The text in this chapter was first published in the journal Frontiers in Aging Neuroscience and was adapted for this thesis. The citation is as follows:

Martinez JL, Zammit MD, West NR, Christian BT, Bhattacharyya A. Basal Forebrain Cholinergic Neurons: Linking Down Syndrome and Alzheimer's Disease. Front Aging Neurosci. 2021 Jul 12;13:703876. doi: 10.3389/fnagi.2021.703876. Erratum in: Front Aging Neurosci. 2021 Aug 05;13:742233. PMID: 34322015; PMCID: PMC8311593.

Abstract

Down syndrome (DS, trisomy 21) is characterized by intellectual impairment at birth and Alzheimer's disease (AD) pathology in middle age. As individuals with DS age, their cognitive functions decline as they develop AD pathology. The susceptibility to degeneration of a subset of neurons, known as basal forebrain cholinergic neurons (BFCNs), in DS and AD is a critical link between cognitive impairment and neurodegeneration in both disorders. BFCNs are the primary source of cholinergic innervation to the cerebral cortex and hippocampus, as well as the amygdala. They play a critical role in the processing of information related to cognitive function and are directly engaged in regulating circuits of attention and memory throughout the lifespan. Given the importance of BFCNs in attention and memory, it is not surprising that these neurons contribute to dysfunctional neuronal circuitry in DS and are vulnerable in adults with DS and AD, where their degeneration leads to memory loss, and disturbance in language. BFCNs are thus a relevant cell target for therapeutics for both DS and AD but, despite some success, efforts in this area have waned. There are gaps in our knowledge of BFCN vulnerability that preclude our ability to effectively design interventions. Here, we review the role of BFCN function and degeneration in AD and DS and identify under-studied aspects of BFCN biology. The current gaps in BFCN relevant imaging studies, therapeutics, and human models limit our insight into the mechanistic vulnerability of BFCNs in individuals with DS and AD.

Introduction

Down syndrome (DS, trisomy 21, T21) is a complex developmental disorder that arises from trisomy of human chromosome 21 (HSA21) (Lejeune, 1959); non-disjunctive trisomy 21 is present in 95% of individuals with DS (Finesilver, 2002, Smith, 2001, Roizen and Patterson, 2003, Bittles and Glasson, 2004), translocations are responsible for about 4% of cases, and trisomy 21 mosaicism occurs in approximately 1-4% (Hamerton et al., 1965, Richards, 1969, Mikkelsen,

1977, Bittles and Glasson, 2004). Intellectual disability in individuals with DS ranges from mild to moderate with deficits in specific domains, including attention and memory. DS features arise because of the uncharacteristic dosage of coding and non-coding sequences found on HSA21. Despite its known cause and high incidence (Presson et al., 2013, Shin et al., 2009, de Graaf et al., 2015), little is known about the underlying developmental defects and degenerative outcomes that cause the characteristics of DS.

DS is also characterized by Alzheimer's disease (AD) pathology that emerges in middle age (Coyle et al., 1986, Scott et al., 1983, Head et al., 2012, Menendez, 2005, Burt et al., 1998, Visser et al., 1997, Snyder et al., 2020). The prevalence of dementia in individuals affected by DS increases with each consecutive decade: 9% between the ages of 45 and 49 years, 18% between 50 and 54 years, and 32% between 55 -59 years with a cumulative risk of 90% by age 65 (Holland et al., 2000, Head et al., 2016, Zigman et al., 1996, Sinai et al., 2018). In addition, the prevalence of symptomatic AD in individuals with DS reaches 90 – 100% by age 70, while only 11% of the general population have AD by the age of 65 (2021, Fortea et al., 2020). Additionally, there are sex differences, with DS males developing AD-like pathology at an earlier age than females (Head et al., 2012, Zigman et al., 1996, Holland et al., 2000). Thus, although the onset of dementia and AD in DS is beginning to be defined, it is not known what triggers the pathology nor what the earliest events in AD in DS are.

Basal forebrain cholinergic neurons (BFCNs) are a vulnerable population of neurons in both DS and Alzheimer's Disease (Yates et al., 1980, Beyreuther and Masters, 1995, Baker-Nigh et al., 2015a, Chen et al., 2018, Salehi et al., 2004). BFCNs provide the primary source of cholinergic innervation to the cerebral cortex, hippocampus, and amygdala, and play a critical role in the processing of information related to cognitive function, as they are directly engaged in regulating

circuits of attention and memory (Ballinger et al., 2016, Mesulam et al., 1983, Woolf, 1991). BFCNs degenerate during aging and cell loss correlates with memory loss in old age and individuals affected by AD (Mori, 1997, White and Ruske, 2002, Mandas et al., 2014). In DS, fewer BFCNs suggest faulty development or increased degeneration as a hallmark of reduced cognition (Casanova et al., 1985). The critical role of BFCNs in cognition, as well as their susceptibility in both DS and AD, provide a clear link to the cognitive decline in both DS and AD.

BFCNS are Important in AD and DS

BFCNs are a Unique Population of Neurons

BFCNs are a cluster of large neurons in the basal forebrain first described in 1872 by Theodor Meynert (Meynert, 1872) and termed the "magnocellular basal forebrain system" (Hedreen et al., 1984) or the nucleus basalis of Meynert (NbM) in primates (Koelliker, 1896). Unlike other neuronal types, whose nuclei of origin are easy to identify, BFCNs often form dense clusters with no easily identifiable borders to justify the identification of a nucleus. Cholinergic neurons have extremely long and complex processes with a single human neuron having an estimated arborization length of >100m (Wu et al., 2014). BFCNs express several neurotransmitter receptors that include adrenergic, glutamatergic, GABAergic, estrogen receptors and endocannabinoids (Mufson et al., 2003, De Souza Silva et al., 2006, Zaborszky et al., 2004, Miettinen et al., 2002, Harkany et al., 2003). Their neuronal projections extend to the cerebral cortex, hippocampus, and amygdala and are the primary source of innervations to the cortex. Unlike primary sensory cortical neurons, cholinergic neurons remodel their axonal arborizations and synapses continually throughout the lifespan (Hasselmo, 2006, Sarter et al., 2003, Botly and De Rosa, 2009, Heys et al., 2010, Schmitz and Duncan, 2018, Mitsushima et al., 2013).

BFCNs are classified based on their projection targets defined in rats and nonhuman primates (Coppola and Disney, 2018, Solari and Hangya, 2018, Mesulam et al., 1983, Butcher and Semba, 1989). Ch1 and Ch2, neurons from the medial septum and the vertical limb of the diagonal band, are the primary source of cholinergic innervation to the hippocampus. Neurons from the horizontal limb of the diagonal band, Ch3, connect to the olfactory bulb, piriform, and entorhinal cortices. These regions act as a network hub for memory, as they are the interface between the hippocampus and neocortex. Neurons in the substantia innominate/nucleus basalis, Ch4, project to the basolateral amygdala and innervate the entire neocortex. Ch1, Ch2, and Ch3 also project to orexin/hypocretin neurons in the lateral hypothalamus region of the brain (Sakurai et al., 2005). The orexinergic nucleus neurons project throughout the nervous system to mediate cognition and various physical processes (Chieffi et al., 2017). The ratio of cholinergic to non-cholinergic neuronal projections in each of these target areas varies and may affect functional connectivity. On average, the ratio is lower in the frontal area (0.3) and higher in the posterior area (0.6) (Zaborszky et al., 2015). Through this complexity, BFCNs regulate attention, memory, learning, and processing of information related to cognitive function, and so deficits in BFCN number or function can negatively impact an individual's spatial reasoning, language, and cognition.

BFCNs in Aging and AD

BFCN dysfunction or degeneration is implicated as a driving factor for disease in a diverse range of human neurocognitive conditions and neuropsychiatric disorders including Parkinson's disease (PD), schizophrenia, drug abuse, and AD (Blanco-Centurion et al., 2007, Détári, 2000, Conner et al., 2003, Goard and Dan, 2009, Jones, 2008, Kaur et al., 2008, Lin and Nicolelis, 2008, Parikh and Sarter, 2008, Weinberger, 2007). The strong correlation between the thinning of the

Ch4 BFCNs and mild cognitive impairment of PD patients (Rong et al., 2021) suggests that loss of BFCNs contributes to the cognitive decline in PD. BFCN expression of histamine H1 receptor (H1R) is decreased in patients with schizophrenia that show negative symptoms and hallmarks of schizophrenia, such as the formation of sensorimotor gating deficit, social impairment and anhedonia-like behavior (Cheng et al., 2021). Deleting the H1R gene in BFCNs in mice is sufficient to elicit these negative symptoms (Cheng et al., 2021), implicating a central role for this gene and these neurons in schizophrenia. Ch1, Ch2, and Ch3 BFCN projections to the orexin/hypocretin nucleus are likely linked to addiction and changes in behavior. Lesioning of BFCNs in mouse models of drug addiction suggest that interactions between BFCN driven individual cognitive-motivational biases and the form of the drug cue encountered are involved in relapse (Pitchers et al., 2017). Together these studies, though limited, support the critical role for BFCN function in circuit function and behavior.

BFCNs undergo a significant level of atrophy during normal aging in mammals, including humans. This age-related degeneration is positively correlated to memory loss in old age and is more prominent in individuals affected by AD (Mori, 1997, White and Ruske, 2002, Mandas et al., 2014). Cholinergic circuits are susceptible to non-pathologic age-related oxidative and inflammatory stress, which stimulates the immune system (Gamage et al., 2020). AD is defined by rapidly accelerated loss of these projection neurons, (Casanova et al., 1985), with up to 90% of nucleus basalis of meynert (NbM) neurons lost in familial cases of AD (Whitehouse et al., 1981). Cholinergic dysfunction correlates strongly with the progression of cognitive decline (Isacson et al., 2002, Nardone et al., 2006). Specifically, BFCN-related cognitive decline involves basocortical projection systems, septohippocampal projection systems and a loss of the high-affinity neurotrophic receptor (TrkA) expression specifically in BFCNs (Mufson et al., 1996,

Ginsberg et al., 2006, Naumann et al., 2002, Mufson et al., 2003, Mufson et al., 2004). The loss of BFCNs during normal aging and in the pathology of AD highlights the importance of these cells in maintaining cognitive function.

Deficits in BFCNs contribute to dysfunctional neuronal circuitry in individuals with DS who have phenotypically unique behavioral patterns in language, attention, and memory. Post-mortem analysis indicates that there are 29% fewer NbM neurons in adult DS compared to controls (Casanova et al., 1985). Fewer BFCNs in older DS patient samples as compared to euploid suggests that loss of BFCNs contributes to memory loss, decreased spatial recognition, and disturbance in language which are common areas of decline in both DS and AD (Davies and Maloney, 1976, Bierer et al., 1995, Mufson et al., 1995, Mufson et al., 1989a, Whitehouse et al., 1982, Ballinger et al., 2016, Perry et al., 1992, Price et al., 1986, Casanova et al., 1985, Coyle et al., 1983, Coyle et al., 1986). The decreased number of these BFCNs in DS may be due to fewer cells established during brain development or due to degeneration. Understanding the vulnerability of these neurons will help us understand the underlying mechanisms of neurodegeneration in both AD and DS.

What is the Mechanism of BFCN Degeneration?

Degeneration of cholinergic neurons in the basal forebrain is strongly correlated with cognitive function. It is not known what causes the degeneration of BFCNs. Several hypotheses have been raised to define the mechanisms underlying BFCN degeneration in DS and AD including those focused on acetylcholine, amyloid-β, tau, inflammation, and retrograde transport (Fig.1). Yet, gaps in our understanding of their role specifically in BFCNs remain.

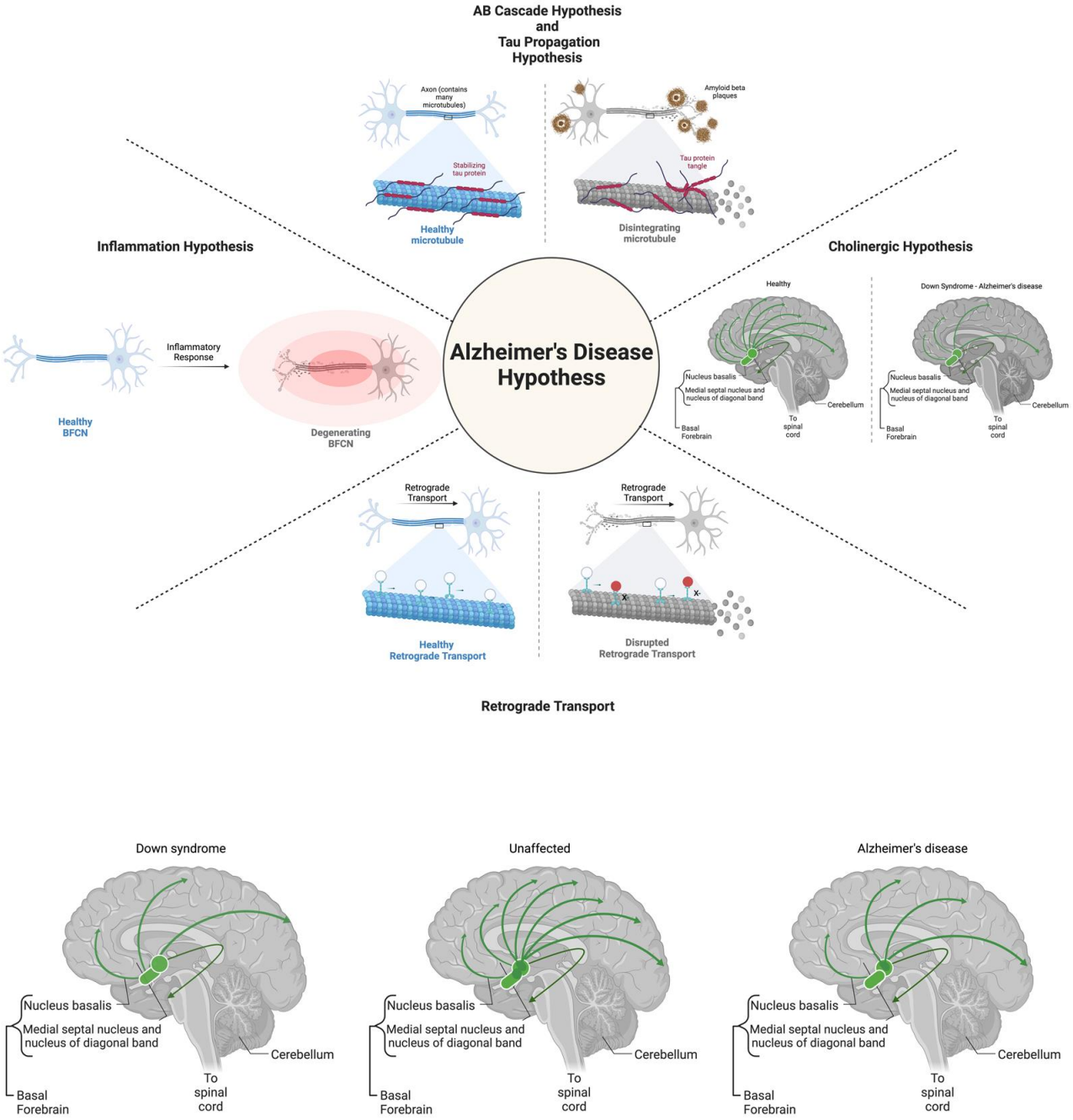

Figure 1. Postulated mechanisms of BFCN degeneration. Created with BioRender.

Cholinergic Hypothesis

Because the cholinergic system is important in various forms of dementia, including AD (Davies and Maloney, 1976, Bierer et al., 1995, Mufson et al., 1995, Mufson et al., 1989a, Whitehouse et al., 1982, Ballinger et al., 2016, Perry et al., 1992, Price et al., 1986), the use of choline acetyl transferase inhibitors to reverse cholinergic hypofunction in AD has been shown to facilitate memory function, albeit to a moderate degree (Ferreira-Vieira et al., 2016). The cholinergic neuronal loss in the basal forebrain is observed not only in AD, but also in PD, DS, Huntington's disease, and other neurocognitive diseases (Arendt et al., 1983, Dubois et al., 1983, Yates et al., 1980, Barron et al., 1987, Kato, 1989, Ferrante et al., 1987, Aquilonius et al., 1975). Studies have demonstrated that cholinergic synapses are affected by amyloid-β oligomers, and this neurotoxicity is the major contributor to cognitive impairment in AD and DS (Terry et al., 1991, Selkoe and Hardy, 2016, Selkoe, 2002). These data and others led to the "cholinergic hypothesis of AD" (Contestabile, 2011, Coyle et al., 1983, Dumas and Newhouse, 2011). As discussed later, this hypothesis has fallen out of favor, but recent data should serve to revive studies on this critical system.

Amyloid-β Cascade Hypothesis

The amyloid-β cascade hypothesis was advanced by the finding of a pathogenic mutation in the APP gene (encoded on HSA21), which indicated that APP metabolism and amyloid-β deposition were the primary events in AD (Hardy and Allsop, 1991, Selkoe, 1991). APP is cleaved by two different proteolytic processes: the amyloidogenic (β pathway, pathogenic) that results in the production of insoluble amyloid-β and the non-amyloidogenic (α pathway, non-pathogenic) pathway (Liu et al., 2019) that does not produce insoluble amyloid-β. It is well established that

high concentrations of amyloid-β protein are neurotoxic to neurons, causing atrophy of the axons and dendrites leading to neuronal death (Yankner et al., 1990). Normally the small amount of amyloid-β that is produced via the β pathway is cleared by the immune system, but APP mutations such as Lys670Asn/Met671Leu (Swedish) can direct more amyloidogenic proteolysis (Yan and Vassar, 2014, Zhou et al., 2018). Similarly, individuals with a rare familial trait known as duplication of APP (Dup-APP), also develop early onset AD (Sleegers et al., 2006, Wiseman et al., 2015, Hooli et al., 2012, Kasuga et al., 2009, McNaughton et al., 2012, Rovelet-Lecrux et al., 2006, Rovelet-Lecrux et al., 2007, Swaminathan et al., 2012, Thonberg et al., 2011). Thus, APP and amyloid-β have a causative role in AD.

The additional copy of HSA21-encoded APP in DS may be a driving factor for the emergence of AD in individuals with DS by increasing amyloid-β. Likewise, individuals with a partial trisomy of chromosome 21 that lack an additional copy of APP do not develop AD (Prasher et al., 1998, Korbel et al., 2009). While these data suggest a key role of APP in the development of AD in DS, recent studies from DS models show the important role of APP in the amyloidogenic aspects of AD but challenge the notion that increased APP levels are solely responsible for DS-associated AD pathogenesis (Ovchinnikov et al., 2018, Wiseman et al., 2018, Wiseman et al., 2015). Additional genes on HSA21 may regulate the course of AD in DS individuals, but further work is required to elucidate their role and importance.

Tau Propagation Hypothesis

The tau propagation hypothesis focuses on the appearance of neurofibrillary tangles (NFTs) and misfolded tau that propagates through the brain in a prion-like way, eventually spreading throughout the brains of AD patients (Frost et al., 2009). Tau proteins stabilize the

microtubules that act as a highway for the transportation of cargo in dendrites and axons (Frost et al., 2009, Clavaguera et al., 2009). Tau is encoded by approximately 352 residues and alternative splicing of exons 2, 3, and 10 results in six isoforms. The balance between isoforms regulates cellular processes (Goedert et al., 1989, Andreadis et al., 1992). An equilibrium between two isoforms (3R and 4R) may be important in preventing the formation of tau aggregates, a common feature in AD pathology. Hyperphosphorylated tau proteins form helical filaments, which aggregate to form NFTs (Nukina and Ihara, 1986, Grundke-Iqbal et al., 1986, Kosik et al., 1986), a pathological feature of AD (Braak and Braak, 1996, Braak et al., 1999).

Individuals with DS show the formation of NFTs as early as 30 years of age (Perez et al., 2019, Lott and Head, 2019, Gomez et al., 2020). Tau phosphorylation and the appearance of NFTs in DS may be regulated by two HSA21 genes, APP and DYRK1A. DYRK1A phosphorylates APP at the Thr668 residue, which leads to an increase in activated APP. The resulting p-APP phosphorylates the Thr212 residue of tau, resulting in pTau that is implicated in AD pathology (Alonso et al., 2018, Alonso et al., 2010). A plasma biomarker study revealed that individuals with DS had decreased amyloid-β over time, while the level of tau and NFT increased leading to a reduction in basal forebrain volume (Schmitz et al., 2020, Mengel et al., 2020). Thus, in addition to APP, tau phosphorylation may be dysregulated in DS and specifically in BFCNs.

Inflammation Hypothesis

Inflammation occurs in the brains of individuals with AD and DS patients as a response to neuritic plaques and NFTs (Wilcock, 2012). The inflammatory response is predominantly mediated by microglial cells, brain-specific macrophages in the central nervous system (CNS) that make up about 15% of all brain cells (Clayton et al., 2017). Microglial inflammatory responses

have been identified as potentially playing an important role in the development of AD pathology (Clayton et al., 2017, Kinney et al., 2018, Chen and Mobley, 2019). In AD patients a 2-5 fold increase in the concentration of aggregated microglia near neurons with NFTs may indicate higher activity of microglial cells in AD (Calsolaro and Edison, 2016). Amyloid-β has a synergistic effect with the cytokine activation of microglia (Meda et al., 1995). It is through the CD36-TLR4-TLR6 receptor complex and the NLRP3 inflammatory complex that amyloid-β can bind to microglia cells, release inflammation factors, and elicit immune responses (Sheedy et al., 2013, Heneka et al., 2013). Levels of inflammatory factors such as TNF-a, IL-1β, TGF-β, IL-12 and IL-8 correlate with AD and increased levels in the CNS have also been implicated in increased damage in brains of AD patients (Michaud et al., 2013). Biomarkers in DS plasma show consistently higher levels of amyloid-β and IL-1β (Startin et al., 2019). Similarly, in DS, IL-1β and TGF-β induce HSA21 proteases ADAMTS1 and ADAMTS5 in the CNS. The ADAMTS proteins are of interest because they are involved in neurodegeneration (Gurses et al., 2016). Inflammation likely plays a major role in AD in DS, but the mechanism is currently under investigation. Additionally, it is not clear whether BFCNs are affected by inflammation.

Retrograde Transport

BFCNs depend on nerve growth factor (NGF) and brain-derived neurotrophic factors (BDNF) for their survival and function (Fahnestock and Shekari, 2019). Both neurotrophic factors are retrogradely transported from BFCN targets. The precursor for NGF (proNGF; pNGF) binds to the NGF receptors TrkA and p75NTR, binding with higher affinity to p75NTR while mature NGF binds more strongly to the TrkA receptor (Fahnestock and Shekari, 2019). BFCNs express both receptors, which are activated by pNGF to elicit TrkA-dependent pathways of survival and

growth through MAPK and Akt-mTOR. However, inactivation or imbalance of TrkA leads to activation of p75NTR-dependent apoptotic pathways, such as JNK. TrkA is also increasingly lost in mild cognitive impairment and AD (Fahnestock and Shekari, 2019). Studies modeling aging with embryonic rat basal forebrain neurons in culture have shown the axonal transport of NGF and BDNF are impaired with age, suggesting a vulnerability of BFCNs in aging as well as in age-related disorders such as AD (Budni et al., 2015).

In DS, it has been speculated that the additional copy of APP on HSA21 has a downstream impact on the retrograde transport of neurotrophins. Using mouse models, overexpression of APP hyperactivates Rab5, a key regulator of endosome fusion and trafficking, leading to abnormally large endosomes, which normally carry the NGF signal retrogradely (Xu et al., 2016). There are two explanations as to how hyperactivation of Rab5 impairs neuronal trophic signaling. First, the enlarged Rab5 endosomes may have a difficult time moving retrogradely within the axon, thus resulting in a net decrease in NGF delivery to the soma (Xu et al., 2018). Alternatively, the increased Rab5 activation can promote premature delivery of trophic signals to late endosomes/lysosomes, resulting in early degeneration of NGF/TrkA signaling (Zhang et al., 2013, Xu et al., 2018). Both possibilities would lead to decreased trophic signaling and support of BFCNs, resulting in neuronal death. No studies have assessed retrograde transport in human DS BFCNs and so we do not know whether similar mechanisms are in play.

Here, we summarize the predominant mechanisms that have been raised that may underlie specifically BFCN pathology. However, other characteristics of trisomy 21 cells may also be important in BFCN pathology. For example, oxidative stress induced by copper-zinc superoxide dismutase (SOD1) has long been implicated in DS (Lott et al., 2006, Perluigi and Butterfield, 2012) and, in fact, treatment of aged Ts65Dn mice with Vitamin E reduced oxidation levels and

decreased cholinergic neuron pathology in the basal forebrain (Lockrow et al., 2009). Human studies are needed to test whether these results translate to humans.

It is also possible deficient autophagy contributes to BFCN pathology (Colacurcio et al., 2018). Assessment of autophagy in human trisomy 21 cortical neurons has recently emerged and identified phosphoinositide PI(3)P as a potential target to correct endosomal dysfunction (Botté et al., 2020). In cortical neurons, a decrease of phosphoinositide PI(3)P was observed in DS fibroblasts, this lack of PI(3)P led to early endosome clustering and ultimately deficits in autophagy (Botté et al., 2020). It will be important for future studies to define vesicle trafficking in BFCNs as well as cortical neurons.

Imaging Reveals Vulnerability of BFCNs in AD and DS

Although there are multiple vulnerable populations of neurons in various brain regions, the classic model of AD pathology progression postulates that the initial accumulation of pTau, and later amyloid-β accumulation, in the entorhinal cortices leads to the degeneration process that spreads to the temporoparietal cortex over time in a stage-like fashion (Braak and Braak, 1991, Thal et al., 2002, Fernandez and Lopez, 2020). Imaging studies of AD models support this hypothesis, as they indicate that the accumulation of pTau and amyloid-β in certain brain regions reflects the local neural vulnerability that spreads over time (Davies and Maloney, 1976, Whitehouse et al., 1981, Arendt et al., 1985, Mesulam et al., 2004, Mattson and Magnus, 2006, Geula et al., 2008, Braak and Del Tredici, 2011, Saxena and Caroni, 2011, Baker-Nigh et al., 2015b, Khan et al., 2014, Grothe et al., 2018, Sepulcre et al., 2018, Hanseeuw et al., 2019). Yet, recent imaging studies have started to challenge this model. A study using cerebrospinal fluid (CSF) and MRI data from the Downs-Alzheimer Barcelona Neuroimaging (DABNI) and Sant Pau

Initiative on Neurodegeneration (SPIN) study that included volumetric T1-weighted MRI sequence, identified a volumetric decrease with age of the basal forebrain of individuals with DS along the AD continuum (Rozalem Aranha et al., 2023). This data significantly correlated with the amyloid, tau, and neurofilament light chain changes in the CSF of individuals with DS and cognitive performance (Rozalem Aranha et al., 2023). Additionally, a longitudinal study using CSF and MRI data from the Alzheimer's Disease Neuroimaging Initiative (ADNI) identified neurodegeneration of the NbM in abnormal and normal groups defined by previously validated CSF pTau/amyloid-β ratios (Fernandez-Cabello et al., 2020). Two non-overlapping and well-powered data sets from the ADNI, along with whole-brain regression models show that the relationship between NbM volumes and neurodegeneration is specific to regions of the entorhinal cortex and the perirhinal cortices (Fernandez-Cabello et al., 2020). These results suggest a model in which amyloid-β pathology in the ascending BFCN projections from NbM first spreads to the entorhinal cortex and then to the temporoparietal neurodegeneration typically attributed to the earliest stages of AD. The degeneration of the BFCN projection system as an early event in AD pathology highlights the susceptibility of these neurons to early pathology and later downstream impacts on other vulnerable populations, challenging the current notion that the entorhinal cortex is upstream of this event. There is a clear need for additional studies exploring the initial events in AD pathology to better understand the early disease stage vulnerable populations.

There are limited human studies in DS that focus on BFCNs as a vulnerable neuronal population, highlighting the critical need for more neuropathological and imaging studies. However, within the last several years, large studies using positron emission tomography (PET) to characterize the preclinical progression of AD in DS have emerged using the AT(N) (amyloid/tau/neurodegeneration) disease research framework (Jack Jr et al., 2018, Rafii et al.,

2020, Fortea et al., 2020). Furthermore, no DS studies with PET focus on imaging of BFCNs during preclinical AD. The Alzheimer's Biomarker Consortium – Down Syndrome (ABC-DS) is an ongoing longitudinal study aimed to better understand AD progression in DS by characterizing AD biomarker change in one of the world's largest DS research cohorts (Handen et al., 2020). With PET imaging, a pattern of early and prominent amyloid-β retention was identified in the dorsal and ventral striatum (Handen et al., 2012); a pattern which has also been observed in other forms of early-onset AD (Bateman et al., 2012, Klunk et al., 2007, Remes et al., 2008, Villemagne et al., 2009). Apart from the striatum, the cortical retention of amyloid-β in DS has an identical pattern to late-onset AD, with amyloid-β increasing at longitudinal rates of 3-4% annually (Lao et al., 2017, Tudorascu et al., 2019, Zammit et al., 2020a, Zammit et al., 2021).

Imaging of NFTs with PET is a more recent addition to the field of AD research, but its use in DS is very limited. Through the Down Syndrome Biomarker Initiative, an early DS study with a relatively small sample size demonstrated that increased NFT burden was highly associated with cognitive impairment (Rafii et al., 2017). A study from the ABC-DS with a large sample size identified that NFT retention in DS conforms to the conventional Braak staging of NFT pathology, with the earliest evidence of NFTs in the entorhinal cortex and hippocampus (Tudorascu et al., 2020). NFT PET studies in DS have also been limited to cross-sectional analyses, and longitudinal measurements are needed to characterize the annual rates of NFT progression and the latency period between the onset of amyloid-β and NFTs. PET imaging of glucose metabolic change is also envisioned as a proxy measurement for neurodegeneration in DS.

In DS, glucose hypometabolism has been observed with local increases in amyloid-β throughout regions implicated in AD (Lao et al., 2018). Glucose hypometabolism in the frontal cortex, anterior cingulate, posterior cingulate, parietal cortex, precuneus and temporal cortex were

also highly associated with worsening cognitive performance evaluated using measures of episodic memory (Zammit et al., 2020b), which have been validated as sensitive indicators of the transition between preclinical and prodromal AD in DS (Hartley et al., 2020). In addition, PET measurement of glucose metabolism was capable of distinguishing cases of MCI-DS and AD from cognitively stable DS, suggesting it as a sensitive marker of neurodegeneration (Zammit et al., 2020b). Increased imaging of AD has shown the progression of biomarkers between DS and late-onset AD are very similar, but future studies would require close examination of BFCNs *in vivo* to identify the link between AD biomarkers and BFCN degeneration.

Interventions for AD and DS

Current FDA-approved pharmacological interventions for AD are limited. There are five approved AD medications; donepezil, galantamine, rivastigmine, memantine and a combination of donepezil and memantine (Association, 2019). Donepezil, galantamine, and rivastigmine are acetylcholinesterase inhibitors, while memantine is a noncompetitive low-affinity NMDA receptor open-channel blocker that also affects glutamatergic transmission (Yiannopoulou and Papageorgiou, 2020). Recent work has focused on designing experimental drugs targeting specific points of the pathophysiological mechanism of AD that include amyloid-β, pTau metabolism, mitochondrial dysfunction, oxidative stress, and inflammation. 298 AD therapies have undergone clinical trials, of which 76 are focused on targeting Aβ peptide or its aggregates (Zhang et al., 2023). These new drugs are classified into four categories that include: 1) focus on reducing Aβ generation, 2) enhancement of degeneration and clearance of Aβ and its aggregates, 3) to neutralize soluble Aβ, or 4) inhibition of Aβ aggregate formation (Zhang et al., 2023). Most, if not all, have proven clinically unsuccessful, with only two antibody-based drugs, aducanumab and

lecanemab, approved by the FDA and 38 discontinued due to ineffectiveness or toxic side effects (Zhang et al., 2023). It is important to note that most of the FDA-approved AD drugs are acetylcholinesterase inhibitors that reduce the breakdown of acetylcholine released from BFCNs. Much like donepezil, galantamine works by inhibiting acetylcholinesterase reversibly and selectively while rivastigmine is a pseudo-irreversible inhibitor of both acetylcholinesterase and butyrylcholinesterase. These inhibitors can mitigate the memory deficits associated with aging and AD (Rusted, 1994, van Reekum et al., 1997, Du et al., 2018). However, their effects appear to be transient, as they only show efficacy during the first year of administration, with further memory decline occurring later. In the AD2000 study, a large "real life" trial on the impact of regular use of donepezil, AD patients treated with donepezil did not show significant benefits compared to placebo in the progression of disability at 3 years of treatment, rendering this approach a symptomatic relief with marginal benefits (Bentham et al., 2004, Du et al., 2018). These results led to the cholinergic hypothesis and targeting of the cholinergic pathway falling out of favor in the AD research community (Cacabelos, 2007). Yet, the cholinergic system is of high importance in AD and DS and that BFCNs remain a relevant cell population and potential therapeutic target.

Despite the prevalence of AD in DS patients, individuals with DS have been traditionally excluded from most clinical trials of anti-dementia drugs (Strydom et al., 2018). Cholinergic therapies have been advocated for DS to ameliorate dysfunctional neuronal circuitry (Kishnani et al., 2001). The available AD-related pharmacologic therapies offer minimal usefulness in symptom reduction and fail to stop or slow down disease progression (Areosa and Sherriff, 2003, Folch et al., 2018, Cacabelos, 2007, Tayebati et al., 2019). Yet, combined treatment with cholinesterase inhibitors and memantine have also been used to ameliorate both cognitive and behavioral issues in AD and DS. A longitudinal study of 310 people with DS and AD indicated

that those undergoing cholinesterase inhibitor treatment had comparable outcomes, improved cognition and behavior, to those with sporadic AD (Eady et al., 2018). More interestingly, individuals with DS treated with either a single cholinesterase or in combination had a median survival rate of ~5.6 years after diagnosis, an improvement compared to those who did not take medication who had a median survival rate of ~3.4 years (Eady et al., 2018). Not only did these results show that modulating the cholinergic system can improve cognition, but it can also have a significant impact on the length of survival for DS individuals diagnosed with AD. Thus, the cholinergic system and BFCNs warrant further investigation as a potential therapeutic target in DS.

In addition to the FDA-approved medications for AD, additional experimental therapies are being considered to ameliorate cognitive decline or AD onset in DS. Inspired by improvement and protective mechanisms against neurodegeneration in *C. elegans* models of PD, treatment of DS induced pluripotent stem cell (iPSC)-derived forebrain neurons with N-butylidenephthalide reduced amyloid-β aggregates and NFTs (Chang et al., 2015). This amyloid-β scavenger is a promising therapy to target the proteopathy of AD that leads to BFCN deficits. Rapamycin rescues molecular pathways associated with abnormal mTOR phosphorylation and ameliorates the rate of neurodegeneration in DS mouse models, improving their cognition (Tramutola et al., 2018). Lastly, the use of Fluoxetine, a widely used antidepressant, in a DS mouse model at an early postnatal age showed promise in increasing neurogenesis and reducing learning deficits (Guidi et al., 2013). However, further human studies focused on BFCNs are needed, as this field heavily relies on animal models.

Modeling AD in DS and BFCNs

Mouse Models: Do they Recapitulate BFCN Pathology?

Mouse models of DS enable experimental approaches that are not feasible in humans, such as the study of disease progression in a regulated environment, intervention trials, validation of imaging results, and also permit gene-gene interaction studies of HSA21-specific DS genes (Herault et al., 2017, Hamlett et al., 2016). Of the 225 protein coding genes found on human chromosome 21, 166 are conserved in three regions in mice, murine (mmu) chromosome 10, 16, and 17 (Akeson et al., 2001, Hattori et al., 2000). Mouse models of DS have provided evidence of the influence of individual genes on HSA21 that lead to deficits in BFCNs (Coyle et al., 1991, Sweeney et al., 1989, Powers et al., 2016, Ash et al., 2014, Cooper et al., 2001, Hunter et al., 2004, Kelley et al., 2019, Kelley et al., 2014a, Powers et al., 2017, Salehi et al., 2006, Kiss et al., 1989).

The vast majority of the aging and AD studies in DS have been conducted on the Ts65Dn mouse, the prevalent model of DS for many years (Davisson et al., 1993, Reeves et al., 1995). Developed in the early 90's by Muriel Davisson, this model contains 120 orthologs of HSA21 protein encoding genes via a segmental trisomy of mmu 16 (Davisson et al., 1993). The aneuploidy in the Ts65Dn mouse is not lethal as in the Ts16 mouse model, but their lifespan is shorter than diploid mice (Sanders et al., 2009). However, 25% of trisomic genes in Ts65Dn are not HSA21 orthologs, and 45% of HSA21 orthologs are not trisomic (Zhao and Bhattacharyya, 2018). Thus, the Ts65Dn model has genetic limitations as an age-related DS and AD pathology model. Nonetheless Ts65Dn mice do show several relevant deficits including progressive memory decline, hippocampal abnormalities, increased APP production, and adult-onset degeneration of BFCNs, locus coeruleus neurons, and noradrenergic cortical innervations (Hamlett et al., 2016). Sex differences have been described in the Ts65Dn model; female Ts65Dn mice show a decrease in BFCN number as well as a smaller NbM region area as compared to males by 34% and 20%

respectively (Kelley et al., 2014b). No human studies have assessed sex differences in BFCNs and so we do not know how well these results translate to humans.

Similar to the Ts65Dn model, the Tc1 mouse model shows many relevant phenotypes including abnormalities in learning, memory, and synaptic plasticity (Gardiner et al., 2003). The Tc1 mouse model is trisomic for 212 of the HSA21 protein coding genes (Hamlett et al., 2016). In contrast to Ts65Dn mice, Tc1 mice also exhibit higher levels of S100B calcium-binding protein, AMPK, and the mTORC1 proteins RAPTOR and downstream kinase P70S6, crucial regulators of cellular metabolism and aging. The Ts1Cje model, which contains a shorter Mmu16 trisomy than the Ts65Dn mouse, and the Ts2Cje model, whose chromosomal rearrangement of the Ts65Dn genome caused a translocation to Mmu12 forming a Robertsonian chromosome, show similar phenotypes to Ts65Dn. Both Ts1Cje and Ts2Cje mice exhibit oxidative stress, tau hyper-phosphorylation, mitochondrial dysfunction, and show some learning and memory deficits, and ultimately BFCN degeneration similar to the processes identified in Ts65Dn mice (Hamlett et al., 2016).

MS1Ts65 mouse models of DS contain only a small fragment of HSA21 orthologs in comparison to other models (Duchon et al., 2011). They contain approximately 33 orthologs of HSA21 genes within the genetic segment ranging from APP to Sod1 (Sago et al., 1998). With complete trisomy of all HSA21 syntenic regions, the Mmu10− Dp(10)1Yey/+ (Ts1Yey), Mmu17− Dp(16)1Yey/+ (Ts2Yey), and Mmu16− Dp(17)1Yey/+ (Ts3Yey) triple aneuploid mouse model is the most complete model of DS to date (Yu et al., 2010, Li et al., 2007). Ts3Yey mice have similar brain morphology to Ts65Dn mice and confirm the genetic basis for behavioral and morphological phenotypes, thus offering promise for developing more appropriate and complete mouse models for DS in the future (Duchon et al., 2011, Hamlett et al., 2016).

BFCN neuropathology is apparent in Ts65Dn mouse models of AD in DS. Age-related degeneration starts around 6-8 months of age, with significant BFCN cell body atrophy at 6 months and major loss at age 8 and 10 months (Hamlett et al., 2016, Contestabile et al., 2006). In addition, major deficits in both choline acetyltransferase (ChAT) and NGF receptor TrkA in BFCNs are detectable at these ages (Contestabile et al., 2006). At a later age, increased neurochemical markers, including inflammatory markers and APP cleavage products, suggest continual progression of AD neuropathy in Ts65Dn mice (Contestabile et al., 2006). Although this study highlights the critical role of BFCNs in the progression of AD in DS, it is crucial to develop both animal and human models that capture the full trisomy in DS to better study this neuropathology.

Targeting the cholinergic pathway as a therapeutic strategy has been carried out in Ts65Dn mice. Maternal choline supplementation and gene expression analysis of laser capture microdissection (LCM)-captured CA1 pyramidal neurons in maternal choline-supplemented Ts65Dn mice offspring at 6 months (before BFCN degeneration) and 11 months (post BFCN degeneration) of age had improved spatial and recognition memory task performance as compared to their littermate controls (Lockrow et al., 2011). These results and others highlight the importance of cholinergic levels in DS for healthy neural circuits (Alldred et al., 2018, Alldred et al., 2019). Memantine treatment in these mice resulted in increased expression of the neurotrophic factor BDNF in the frontal cortex and hippocampus (Lockrow et al., 2011). Thus, mouse models of DS provide proof-of-principle that cholinergic therapies may be successful.

Human Models

The incomplete genetic recapitulation of HSA21 in mouse models, and trisomy of non-HSA21 orthologs, likely influences the effects of orthologous HSA21 genes and, more

importantly, may cause genetic consequences and downstream cellular and behavioral characteristics that are not relevant to DS. Thus, there is need for analysis of human cells from individuals with DS to have complete trisomy of HSA21 (Herault et al., 2017, Zhao and Bhattacharyya, 2018). In addition, the failures of clinical trials in AD for therapeutic targets based on mouse models argues for the use of human patient-derived cells in target identification and drug screening.

The discovery of reprogramming factors to generate iPSCs from adult somatic cell types opened the doors to derive PSCs from individuals with specific genetic and non-genetic disorders, including DS (Takahashi et al., 2007, Thomson et al., 1998, Yu et al., 2007, Park et al., 2008b). iPSCs can model human neural development by mimicking *in vivo* spatial and temporal cues during brain development *in vitro* (Tao and Zhang, 2016). and enable the establishment of functionally specialized neural subtypes (Park et al., 2008a, Mou et al., 2012, Chou et al., 2012, Briggs et al., 2013, Chen et al., 2014, Lu et al., 2013, Pipino et al., 2014, MacLean et al., 2012, Weick et al., 2013b, Hibaoui et al., 2014, Jiang et al., 2013, Huo et al., 2018b). iPSCs are thus a useful model system for studying DS and AD (Ovchinnikov et al., 2018, Murray et al., 2015a, Li et al., 2012, Real et al., 2018).

Isogenic control iPSCs are important research tools to distinguish the consequences of trisomy 21 from human genetic variation. The generation of isogenic euploids can result from culture-induced spontaneous loss of the extra HSA21 (Park et al., 2008a, MacLean et al., 2012). Alternatively, 1-4% of DS cases are mosaic individuals in which their somatic cells are mosaic for T21 (Papavassiliou et al., 2009, Murray et al., 2015b). By taking advantage of cellular mosaicism, isogenic T21 and euploid iPSCs can derived from the same individual (Gough et al., 2020, Murray et al., 2015a, Weick et al., 2013b).

Isogenic cells can be generated by inducing chromosome loss (Real et al., 2018). In addition, various methods have been used to genetically correct the gene dose of the T21 by eliminating or selectively mutating specific genes. Alternatively, others have taken a candidate gene approach to selectively reduce the gene dose using CRISPR/Cas9-mediated gene manipulation. Full chromosomal correction of the gene dose imbalance has been accomplished using XIST-mediated silencing of trisomic chromosome 21 (Jiang et al., 2013), TKNEO-induced loss of the trisomic chromosome 21 (Li et al., 2012), and ZSCAN-induced elimination of the extra chromosome 21 in iPSCs (Amano et al., 2015). These strategies may enable the elucidation of the genetic and cellular consequences of T21.

iPSC to BFCN

Much of the research done using T21 iPSCs derived from individuals with DS has been to understand cortical development and pathology (Huo et al., 2018b, Weick et al., 2013b, Real et al., 2018). T21 iPSC-derived cortical neurons showed impairment in synaptic activity, as well as compensatory responses to oxidative stress (Weick et al., 2013b, Briggs et al., 2013, Sobol et al., 2019, Shi et al., 2012).

Little work has been done to model BFCNs with iPSCs in DS and AD. Basal forebrain neurons (including BFCNs and GABAergic interneurons) originate in neurogenic areas of the most ventral regions of the telencephalon, the medial ganglionic eminences (MGE) and preoptic area (POA) (Sussel et al., 1999, Brazel et al., 2003). Patterning of the MGE is dependent on a sonic hedgehog (SHH) signaling gradient for ventralization of the neural tube (Li et al., 2009, Gulacsi and Anderson, 2006, Xu et al., 2005). MGE progenitors express the transcription factor NKX2.1, whose expression is regulated by SHH (Xu et al., 2010, Xu et al., 2008, Du et al., 2008). Despite

the known development of BFCNs from the MGE, few differentiation protocols have been established to generate BFCNs from hPSCs (Bissonnette et al., 2011, Duan et al., 2014, Hu et al., 2016, Liu et al., 2013a), and many result in mixed populations of cells. The most robust technique (Hu et al., 2016, Liu et al., 2013a) relies on an initial ventralization patterning with SHH and addition of NGF to allow for the survival, differentiation and maturation of BFCN and yields ~90% progenitors expressing NKX2.1. Moreover, ~40% of NKX2.1+ cells co-express OLIG2 and ~15% of NKX2.1+ cells also express ISLET1, which are ventral markers and are both important for BFCN development (Furusho et al., 2006, Wang and Liu, 2001). ~40% of the resulting neurons express Choline Acetyltransferase (ChAT), the enzyme responsible for the biosynthesis of the neurotransmitter acetylcholine and a mature BFCN marker (Hu et al., 2016). Others have also successfully used small molecules to pattern BFCNs resulting in efficiencies ranging from 15% up to 80% (Yue et al., 2015, Liu et al., 2013b, Hu et al., 2016, Muñoz et al., 2020). Although the yields of BFCNs are good, the mixed neuronal cultures leave room for improvement in the established BFCN protocols.

The promising strategies to derive BFCNs from PSCs and the use of isogenic iPSCs will enable us to define markers of dysfunction, aging and degeneration in these cells to reveal molecular signatures and signaling pathways underlying BFCN degeneration in DS and DS-AD. One of the many advantages of PSC models is the retention of the human genetic background by establishing patient specific iPSCs (Brazel et al., 2003, Li et al., 2009). However, through the reprogramming process iPSCs lose many of the aging markers of the somatic donor cells (Xu et al., 2005, Gulacsi and Anderson, 2006). The resulting iPSCs also share transcriptional and functional profile similarities to those in fetal development, making it difficult to study age-related diseases. Thus, generating hPSC-derived neurons that mirror those in the adult and aging brain is

essential for neurodegenerative disease modeling using hPSCs. Further, the results have the potential to inform our understanding of the vulnerability of BFCNs in DS, AD, PD (Brazel et al., 2003), amyotrophic lateral sclerosis (Li et al., 2009), progressive supranuclear palsy (Xu et al., 2005, Gulacsi and Anderson, 2006), and olivopontocerebellar atrophy (Xu et al., 2010).

Summary

DS is observed in approximately one in every 700 live births (Finesilver, 2002, Smith, 2001, Roizen and Patterson, 2003), making DS the most common cause of intellectual disability. With the increased life expectancy of DS individuals, it is important to study the cellular and molecular mechanisms that underlie neurodegeneration and AD in DS (Baker and Petersen, 2018, Duncan, 2011, Sawada et al., 2008). Here, we have raised the need to address the significant gaps in the understanding of the vulnerability of BFCNs in aging and disease by highlighting the critical role of BFCNs in cognition, the vulnerability of BFCNs in animal models of DS and AD and indications that BFCN degeneration may be one of the earliest events in AD and DS neuropathology.

Funding

This work was supported by NIH grants R03HD083538 and funding from UW-Madison, the Wisconsin Alumni Research Foundation, the Wisconsin Partnership Program New Investigator Program, and the Jérôme Lejeune Foundation to A.B. J.M. was supported by the UW–Madison Biotechnology Training Program (NIH 5 T32 GM135066). This study was supported in part by a core grant to the Waisman Center from the National Institute of Child Health and Human Development (U54 HD090256).

CHAPTER TWO: A HUMAN STEM CELL MODEL OF BASAL FOREBRAIN CHOLINERGIC NEURONS

Jose L. Martinez[1,2], Andrew J. Petersen[2], Ke Xu[2], Zhong-Wei Du[2], Su-Chun Zhang[2,3,4], Anita Bhattacharyya[2,5]

[1]Cellular and Molecular Biology Graduate Program, University of Wisconsin, Madison, Wisconsin

[2]Waisman Center, University of Wisconsin, Madison, Wisconsin

[3]Department of Neuroscience, School of Medicine and Public Health, University of Wisconsin, Madison, Wisconsin

[4]Department of Neurology, School of Medicine and Public Health, University of Wisconsin, Madison, Wisconsin

[5]Department of Cell and Regenerative Biology, School of Medicine and Public Health, University of Wisconsin, Madison, Wisconsin

*** Correspondence:**

Anita Bhattacharyya, Ph.D.

bhattacharyy@waisman.wisc.edu

Abstract

Basal forebrain cholinergic neurons (BFCNs) are a neuronal population critical for memory and cognition. BFCNS are vulnerable to degeneration in diseases such as Alzheimer's Disease (AD) and Down syndrome-related Alzheimer's Disease (DS-AD). The key molecular pathways involved in BFCN degeneration are poorly understood, but *in vitro* generation of BFCNs from human pluripotent stem cells (hPSCs) is a powerful method by which to investigate and identify these factors. However, few differentiation protocols have been established to derive BFCNs from hPSCs.

To address this methodological gap, we used a human embryonic stem cell line, H9, to develop a protocol that mimics key transcription factor expression in neural progenitor cells (NPCs) as they differentiate into BFCNs. BFCNs develop from LHX8 and ISLET1-expressing progenitors in the ventral telencephalon and ultimately express ChAT upon maturation. We first modified existing protocols with the addition of SHH and validated that the early addition of SHH results in robust NKX2.1 expression, indicative of ventralization. BFCN progenitor fate specification (LHX8 expression) was accomplished by the addition of NGF at different time points. Results showed a time-dependent increase in the expression of LHX8, suggesting emergence of a BFCN identity. We also found that the addition of BMP9 results in an increase in ISLET1 expression, indicating a progression toward mature BFCNs. Together these results provide a new strategy to differentiate hPSCs to BFCNs.

Introduction

Since the first isolation of human embryonic stem cell (hESC) lines in 1998, they have proven to be a useful tool to recapitulate human embryonic development and derive neuronal cell types using specific signaling cues (Thomson et al., 1998). In 2001 Dr. Zhang, derived the first

human neural cells from hESCs, and showed that these pluripotent cells could be pushed to a neuronal lineage and plated to form self-organizing neuronal rosettes that resemble features of the embryonic neural tube and eventually generate neurons (Zhang et al., 2001). In addition to hESCs, the subsequent discovery of reprogramming factors to generate induced pluripotent stem cells (iPSC) from somatic cell types (Yu et al., 2007, Takahashi et al., 2007, Park et al., 2008b) has allowed for greater access to derive cells from individuals with specific diseases. Induced pluripotent stem cells have been used to study different development paradigms (Zhang et al., 2001, Shi et al., 2017). These and subsequent studies show that both hESCs and iPSCs can be used as powerful tools to study human neural subtypes using similar spatial and temporal cues as seen *in vivo* (Hu et al., 2010).

Basal forebrain cholinergic neurons (BFCNs) are a neuronal population critical for memory and cognition. They are vulnerable to degeneration in disease (i.e. AD and DS-AD) (Geula et al., 2021), yet key molecular pathways involved in BFCN degeneration are poorly understood. However, *in vitro* generation of BFCNs from human pluripotent stem cells (hPSCs) is a valuable method by which to investigate and identify these factors. Nonetheless, few differentiation protocols using developmental cues and no enrichment/filtering steps have been established to derive BFCNs from hPSCs (Bissonnette et al., 2011, Hu et al., 2016).

Developing strategies to differentiate specific neuronal subtypes depends on foundational knowledge of essential developmental processes (Kim, 2015, Zhao and Bhattacharyya, 2018). Basal forebrain neurons, BFCNs and GABA interneurons originate from the most ventral regions of the telencephalon, medial ganglionic eminences (MGE), and preoptic area (Sussel et al., 1999). In particular, the MGE is the source of cholinergic neurons, interneurons that migrate to the cortex, other neurons, and glia for the hippocampus (Brazel et al., 2003). Development and patterning of

the MGE rely on an increased Sonic hedgehog (SHH) signaling gradient and a decrease in WNT signaling gradient for the ventralization of the neural tube (Williams and Riedemann, 2021). To facilitate this regionalization the administration of SHH can be added during *in vitro* neural induction (Watanabe et al., 2005, Li et al., 2009, Liu et al., 2013b, Maroof et al., 2013, Nicholas et al., 2013). NKX2.1 is a transcription factor that is a specific to MGE progenitors, and it is regulated by SHH (Gulacsi and Anderson, 2006). In addition to NKX2.1, several transcription factors have been associated with the differentiation of BFCN, including LHX8 and ISLET1. In a mouse knockout of LHX8 model, BFCNs showed a reduction while other neuronal subtypes did not see the same outcome (Mori et al., 2004, Zhao et al., 2003, Fragkouli et al., 2005), linking LHX8 as a pivotal factor for the development of cholinergic neurons (Manabe et al., 2007). LHX8, a LIM-family homeodomain transcription factor, has been implicated as the subsequent step in the development of BFCNs after patterning to the MGE as cells that express LHX8 become cholinergic neurons (Matsumoto et al., 1996, Asbreuk et al., 2002, Nóbrega-Pereira et al., 2008). Furthermore, LHX8 has been shown to interact with ISLET1 (Cho et al., 2014), leading to the modulation of the expression of cholinergic phenotypes leading to mature ChAT-expressing BFCNs.

Although the development of BFCNs from the MGE has been well established from neurodevelopmental studies (De Carlos et al., 1995, Allaway and Machold, 2017), few differentiation protocols are available to generate BFCNs from hPSCs (Krajka et al., 2021, Du et al., 2015, Muñoz et al., 2020, Duan et al., 2014). Some of the most established cholinergic neuron differentiation protocols were developed by Drs. Zhang (Du et al., 2015) and Muñoz (Muñoz et al., 2020). Both protocols rely on an initial ventralization patterning with SHH and addition of NGF to allow for the survival, differentiation, and maturation of BFCNs. Dr. Zhang's protocol

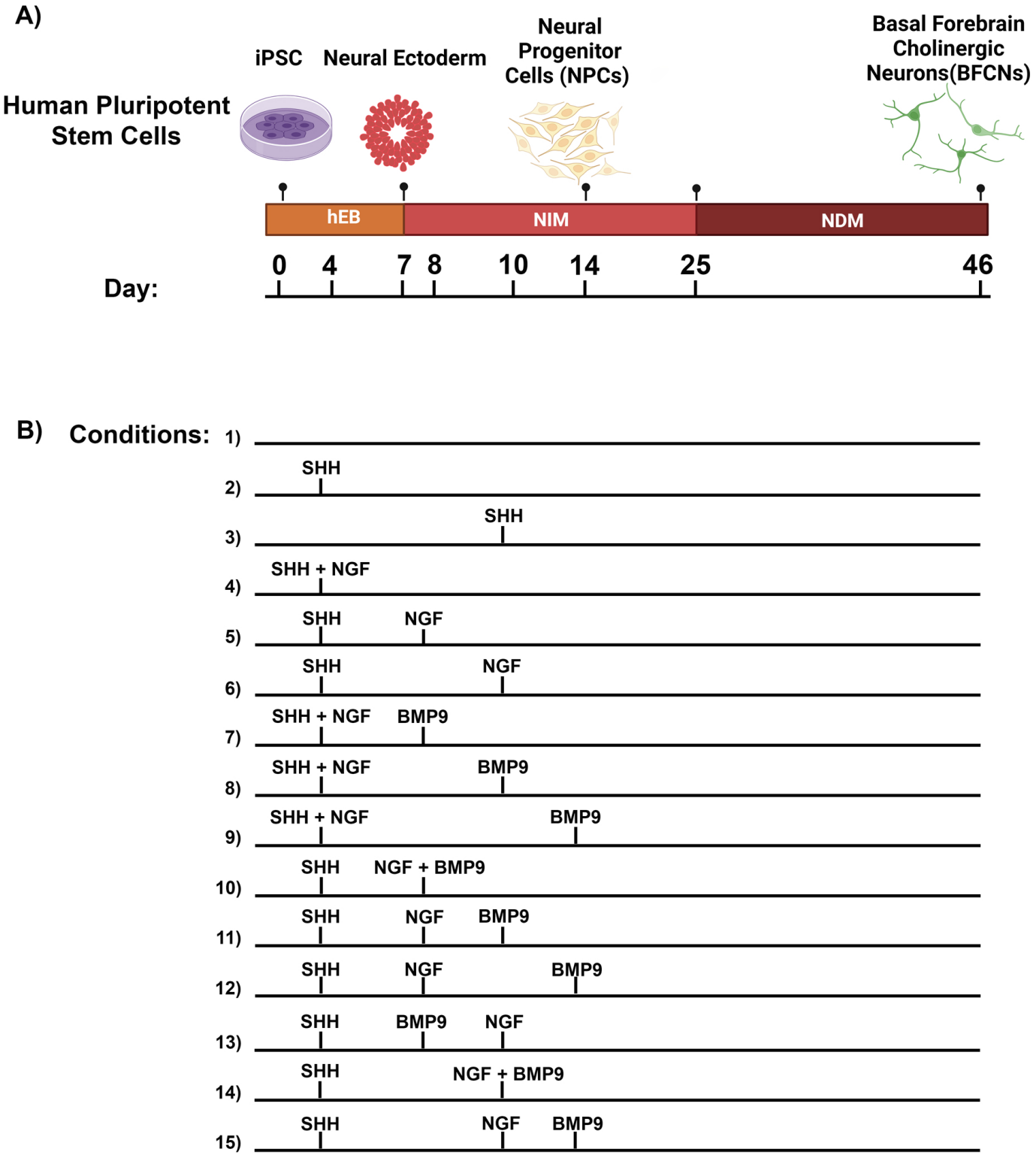

Figure 1. Experimental Design. A) Differentiation stages and time points. B) Experimental conditions located at the time points they will be added.

yields 90% progenitors expressing NKX2.1 of which ~40% co-express OLIG2 and ~15% also express ISLET1, which are ventral markers and are both important for BFCN development (Du et al., 2015). By day 35 approximately 40% of the neurons express Choline Acetyltransferase (ChAT), the enzyme responsible for biosynthesis of the neurotransmitter acetylcholine and a mature BFCN marker. While Dr. Muñoz's protocol utilizes five small molecules (FGF-2, FGF-8, 5FdU, BDNF, and BMP9) in addition to SHH and NGF, resulting in ~80% of β-Tubulin positive cells expressed ChAT (Muñoz et al., 2020).

Here we aim to develop a more efficient protocol to produce BFCNs derived from human pluripotent stem cells. To do so, the effectiveness of SHH addition at various time points was evaluated to identify the most efficient time point for the upregulation of the MGE specific marker NKX2.1, indicating an effective ventralization of hPSCs. Nerve Growth Factor (NGF) and Bone Morphogenetic Protein 9 (BMP-9) addition at several time points and combinations in conjunction with the optimal time point addition of SHH was assessed to identify the optimal combination that enhanced the expression of LHX8 and ISLET1 in NPC respectively, indicative of a BFCN cell fate. We provide a new differentiation protocol for BFCNs derived from hPSCs that will enable mechanistic studies on BFCN susceptibility in disease.

Results

Differentiation of Human Pluripotent Stem Cells to MGE progenitors

A human embryonic stem cell (hESC) line and a human induced pluripotent stem cell (iPSC) line were used. The human embryonic stem cell line, H9-Chat-Cre-AAVS1-Pur-hM3Dq-mCherry, was a donation from the Su-Chun Zhang lab at the University of Wisconsin – Madison. The human induced pluripotent stem cell line, WC24B-Chat-P2A-Cre AAVS1-mCherry, was generated using a CRISPR/Cas9 approach.

Human Pluripotent Stem Cells were maintained on mouse embryonic fibroblasts (MEF) feeder plates and kept in a humidified incubator at 37 °C, and 5% CO_2. After manually marking and removing differentiating colonies, they were fed daily with hESC media (DMEM/F12, 20% knockout replacement serum, nonessential amino acids, L-glutamine, β-mercaptoethanol, and FGF-2). Cells were passaged every 6 – 7 days by incubating with 1 mg/mL of Collagenase (Gibco) for 5 min at 37 °C. After inspecting the cells and verifying that the borders of the colonies started to detach from the plates, hPSCs were washed with hESC media and colonies were mechanically detached and transferred to a conical centrifuge tube. Collected colonies were centrifuged for 1 min at 1000x g, the supernatant was removed, and colonies were resuspended in hESC before being replated at a 1:5 or 1:6 ratio. The hPSC cultures were tested regularly for mycoplasma.

Human Pluripotent Stem Cells were differentiated in a 2D culture system (Fig. 1A) to test fifteen media conditions to optimize the differentiation protocol to BFCNS (Fig. 1B). The first three conditions entailed the addition of SHH at day 4 or day 10, and the control condition of no addition of SHH which served as our comparison group. The next three conditions contained SHH addition at day 4 in combination with the addition of NGF at either day 4, day 8, or day 10, and each combination was compared to the addition of SHH at day 4 with no addition of NGF. The final nine conditions involved the addition of SHH at day 4 and NGF at either day 4, day 8, or day 10, in combination with BMP-9 addition at either day 8, day 10, or day 14. Three technical replicates were carried out for each experiment.

SHH is a vital signaling peptide that induces the expression of NKX2.1 which is crucial for the patterning and specification of the MGE *in vivo* (Xu et al., 2008). Homeodomain-containing transcription factor *NKX2.1* is required for the specification of MGE progenitors (Sussel et al., 1999). Others have previously patterned to the MGE by the addition of SHH at day

10 (Krajka et al., 2021, Liu et al., 2013b, Giffin-Rao et al., 2022). To determine the optimal *in vitro* administration of SHH we evaluated two different ventralization timepoints, day 4 and day 10. Gene expression analysis via qPCR of *NKX2.1* was performed at day 17 which revealed a significant increase in the expression of *NKX2.1* at both time points when compared to no addition of SHH (Fig. 2B). We anticipated that ventral makers would follow a temporal dosage effect, a longer and earlier exposure to SHH would result in a higher expression level. The addition of SHH on day 4 led to a higher expression of *NKX2.1* when compared to no addition of SHH, leading to a 1.39 Log2Fold Change. While addition of SHH on day 10 led to an increase in *NKX2.1* expression of 0.55 Log2Fold Change. These results suggest that the necessary neural precursor population needed to derive BFCNs is enriched when ventralization is initiated earlier in neural induction, validating previous studies showing a temporal dosage effect of SHH on NKX2.1 and other ventral markers. Results suggest that the day 4 addition of SHH is an optimal time point for this differentiation protocol.

Enrichment of BFCN Precursor Cells Expressing LHX8

LHX8 is a specific transcription factor expressed in BFCN neural precursors (Mori et al., 2004, Manabe et al., 2007, Elshatory and Gan, 2008). NGF activates TrkA, which in return increases LHX8 levels that lead to the production of TrkA, resulting in a positive feedback loop between NGF, TrkA, and LHX8 (Tomioka et al., 2014). To determine the optimal timing of NGF and enhance the BFCN precursor population expressing LHX8, we evaluated three different timepoints (day 4, day 8, or day 10). To assess the timing of NGF addition (day 4, day 8, or day 10), qPCR of *LHX8* was performed at day 17. The addition of NGF had a clear effect on the expression of *LHX8*, with a significant difference observed in the Log2Fold Change at day

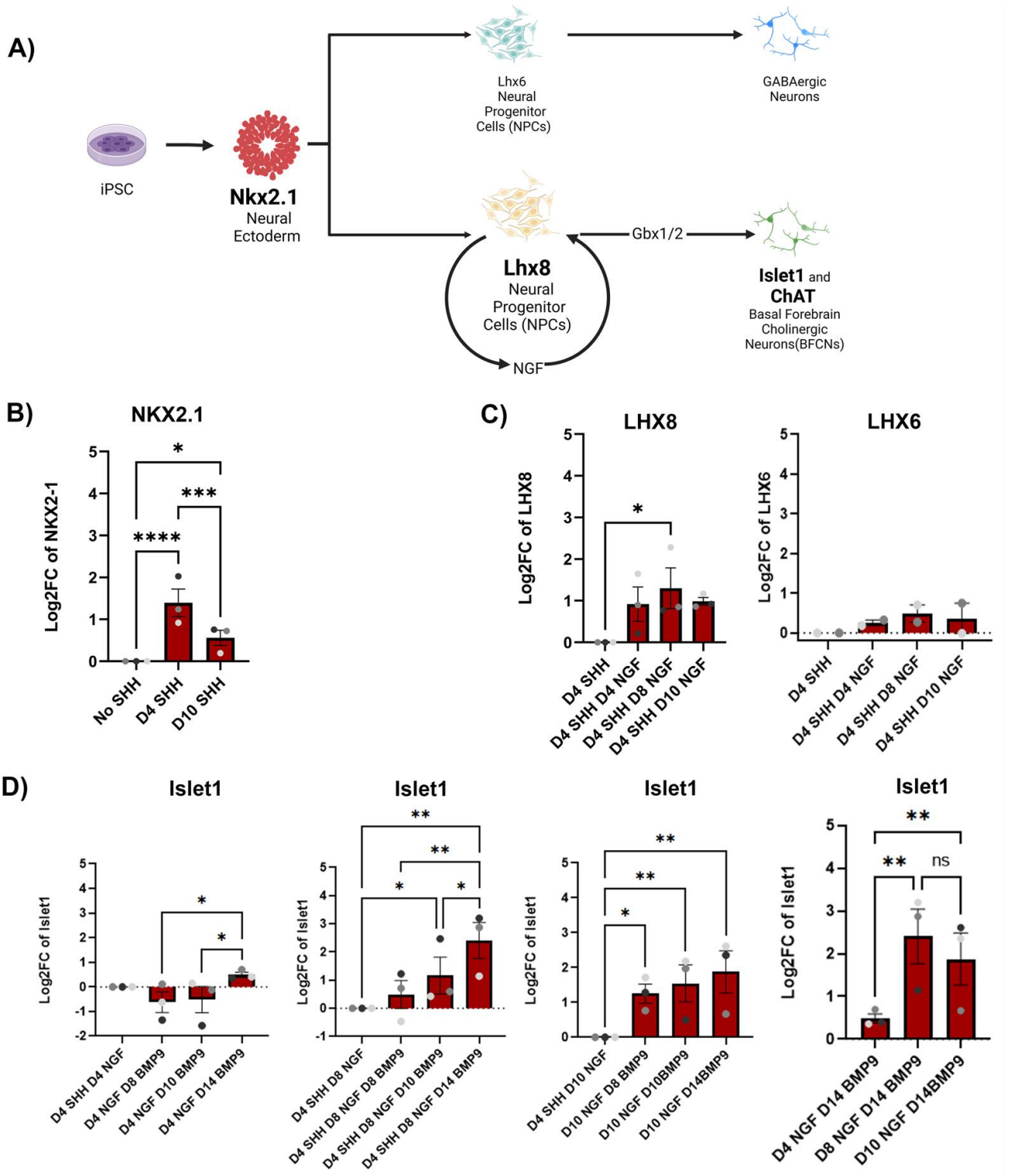

Figure 2. Stepwise Enrichment of BFCN Precursor Cells. A) iPSC differentiation stages leading to BFCNs and GABA neurons. B-D) qPCR of NKX2.1, LHX6, LHX8, and ISLET1 post addition of small molecules at various time points. Statistical significance was determined by a one-sample t-test on Log2Fold Change values.

8 when compared to no addition of NGF (Fig. 2C). LHX6 expression in the MGE is essential to GABA interneuron maturation and migration (Neves et al., 2013). The addition of NGF at all time points increased the expression of *LHX6*, however, no significant difference was observed in expression when compared to no addition of NGF. Taking these results together, NGF increases the overall expression of both *LHX6* and *LHX8*, however the addition of NGF at day 8 showed a significant difference in the expression of *LHX8* and not *LHX6*.

Enrichment of BFCN Precursor Cells Expressing ISLET1

ISLET1 is critical for the maturation of BFCNs, and BMP-9 signaling has been shown to upregulate genes in the BFCN transcriptome that are critical for the maturation of BFCNs (Lopez-Coviella et al., 2005). Bissonnette et al. have cautioned that the timing of BMP-9 is crucial as incorrect addition can result in neurons that do not express BFCN-specific markers (Bissonnette et al., 2011). To determine the optimal timing of BMP-9, to enhance the BFCN precursor population expressing *ISLET1*, and subsequently the efficiency of our protocol, we assessed the timing of a three-day BMP-9 pulse starting at day 8, day 10, or day 14. *ISLET1* qPCR was performed on day 17. The addition of BMP9 in combination with NGF showed a time-dependent increase in the Log2FoldChange of *ISLET1* irrespective of what NGF timepoint the BMP-9 was combined with (Fig. 2D). BMP-9 timepoint addition with NGF at day 4, day 8, and day 10 all showed significant differences when compared to the no BMP-9 addition condition (Fig. 2D). Comparing the Log2FoldChange of *ISLET1* between the BMP-9 day 14 addition with NGF at day 8, day 10, or day 14, revealed that the addition of NGF at day 8 and BMP-9 pulse at day 14 had the highest statistically significant difference (Fig. 2D). These results suggest that the later addition of BMP-9 is best for the expression of key markers for BFCNs and

identifies the combination of NGF at day 8 and BMP-9 at day 14 as optimal for the enrichment of BFCN precursor cell population.

Terminal Differentiation of ChAT-Expressing Basal Forebrain Cholinergic Neurons

Once an efficient patterning protocol for the generation of BFCNs was identified, the identity of mature neurons was qualitatively determined by immunofluorescence analysis. Approximately 45 days after initiating neural differentiation to BFCNs, neurons were stained with NeuN, β-Tubulin, Choline acetyltransferase (ChAT) or mCherry, and DAPI. CHAT is responsible for the biosynthesis of the neurotransmitter acetylcholine, from acetyl-coenzyme A and choline (Cho et al., 2014). Consequently, it is essential for the formation of cholinergic interneurons. Our resulting combined (H9-Chat-Cre-AAVS1-Pur-hM3Dq-mCherry and WC24B-ChAT-P2A-Cre-AVVS1-mCherry) efficiency was 63%, while H9-Chat-Cre-AAVS1-Pur-hM3Dq-mCherry had an efficiency of 57% and WC24B-ChAT-P2A-Cre-AVVS1-mCherry had an efficiency of 72% (Fig. 3C). These results suggest we developed an efficient protocol to generate mature BFCNS form hPSCs.

Materials and Methods

Human Pluripotent Stem Cells

We used two human pluripotent stem cell (hPSC) lines, a human embryonic stem cell (hESC) line, and a human induced pluripotent stem cell (iPSC) line. The human embryonic stem cell line, H9-Chat-Cre-AAVS1-Pur-hM3Dq-mCherry, was a donation from the Su-Chun Zhang lab at the University of Wisconsin – Madison. This line was used in the preparation of an enhanced BFCN protocol, for its ease in validation of ChAT-expressing neurons (Fig. S1).

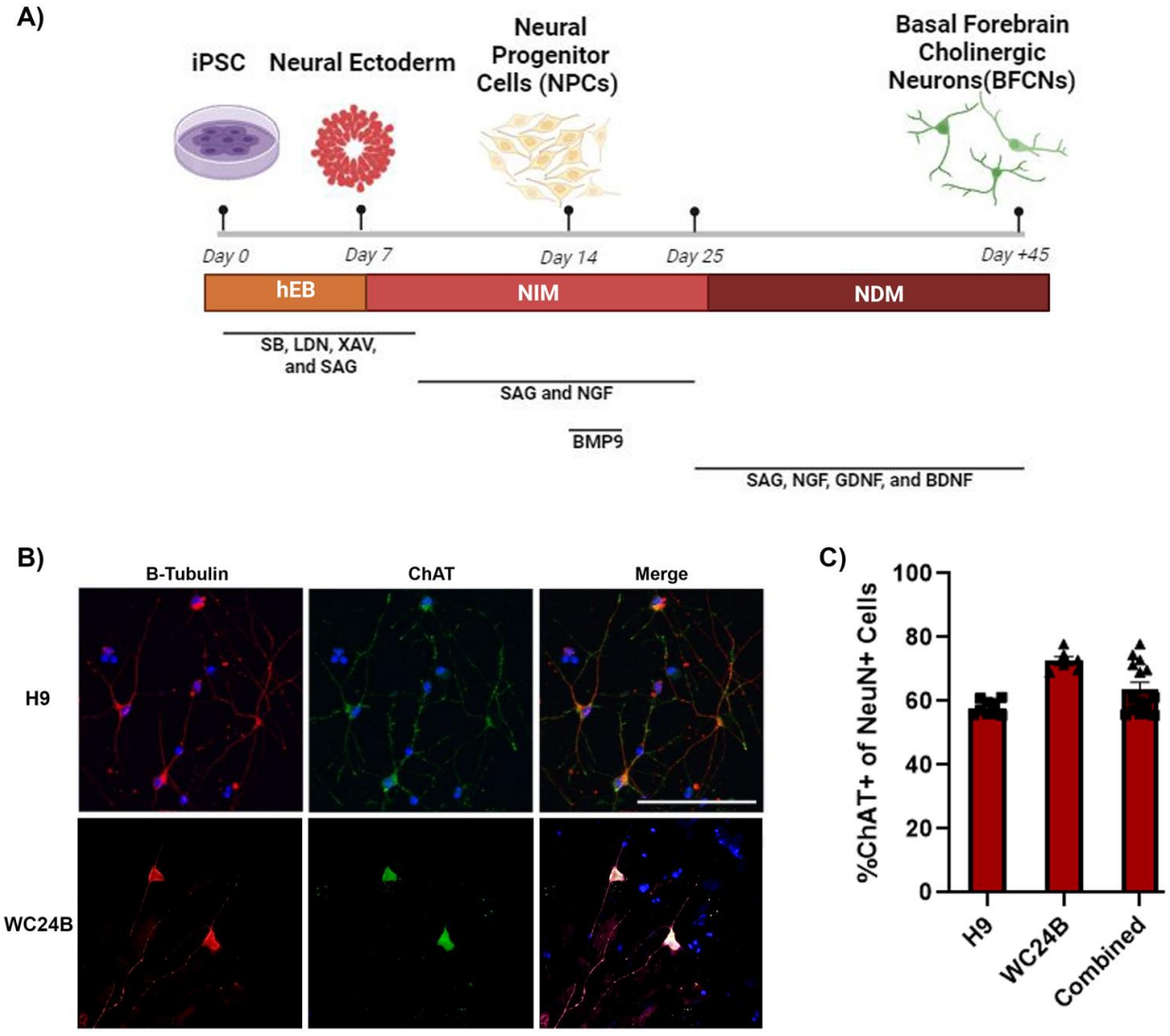

Figure 3. Differentiation to BFCN with Enhanced Protocol. A) Schematic of enhanced BFCN differentiation protocol. B) Qualitative validation of the generation of BFCNs via immunostaining with ChAT. C) Quantification of differentiation efficiency to BFCNs.

The iPSC line, WC24-02-DS-B, was acquired by working closely with clinicians at the University of Wisconsin – Madison Waisman Center, as fibroblasts from an individual were reprogrammed as previously described (Giffin-Rao et al., 2022). WC24-02-DS-B was used to generate a ChAT reporter line. WC24B-Chat-P2A-Cre AAVS1-mCherry, was generated using a CRISPR/Cas9 approach. A day before electroporation iPSCs were cultured in hESC medium on MEFs with Rho Kinase (ROCK)-inhibitor (1.0mM, Calbiochem, H-1152P). Cells were digested by TrypLE express Enzyme (Life Technologies) for ~4 min, washed with DMEM/F12, and pelleted. Cells were dispersed into single cells in BioRad Electroporation Buffer, and $2x10^6$ cells were electroporated as previously described (Chen et al., 2015) in 500 mL of Electroporation Buffer (KCl 5mM, MgCl2 5mM, HEPES 15mM, Na2HPO4 102.94mM, NaH2PO4 47.06mM, PH=7.2) using the Gene Pulser Xcell System (Bio-Rad) at 250v, 500μF in 0.4 cm cuvettes (Phenix Research Products). Electroporation took place in a cocktail of 15 mg of the pLentiCRISPR-V2 plasmid (Adgene, Cat. #52961), two ChAT gRNAs, and 30 ug of NF1X-P2A-CRE-FRT-Puro donor plasmid. Post electroporation, cells were plated on MEFs in 1.0 mM ROCK inhibitor and cells were selected with puromycin (0.33 ug/mL, Invivogen, ant-pr-1) as previously described by Steyer (Steyer et al., 2018). Concurrent with puromycin treatment, the cells were fed with MEF-conditioned hESC media containing 1 uM ROCK inhibitor and 1:5,000 FGF-2. After removal of the puromycin at 96 hours, cells were cultured in MEF-conditioned hESC and FGF-2 (1:5,000) until colonies were visible.

Differentiation Conditions to Basal Forebrain Cholinergic Neurons

Differentiation for all experimental conditions was initiated by detaching hPSC colonies with dispase to form aggregates known as embryoid bodies (EBs) at day 0. For the first 4 days,

EBs were fed daily with hEB media (DMEM/F12, 20% knockout replacement serum, nonessential amino acids, L-glutamine, and β-mercaptoethanol) containing dual SMAD inhibitors (SB-431542 and LDN-193189 2HCl). Starting from day 4 until day 7, EBs were maintained in NIM (DMEM/F12, N2 supplement, nonessential amino acids, and heparin) and fed every other day for 3 days. EBs were attached to plates on day 7 using NIM and 5% FBS for approximately 6 – 8 hours before the media was replaced with NIM. From day 7 until approximately day 14 plated EBs were fed every 2 – 3 days with NIM until neural rosettes emerged. At this point, EBs were detached using a P1000 pipette and grown in suspension as neurospheres (NS) and maintained in NIM media. Half of the NS media is replaced with fresh NIM every 3 – 4 days until day 25 when they are dissociated with Accutase (Sigma) and plated on coverslips coated with Poly-L-ornithine hydrobromide (Sigma) and laminin (Invitrogen) at a density of 25,000 – 30,000 cells per coverslip. Plated neurons were fed the following day with 500 uL of NDM with CompoundE (Millipore), after which half media changes happened every 3 – 4 days with fresh NDM until they are ready to analyze three weeks post-plating.

qPCR

Isolation of total RNA from three technical replicates of differentiation was done at day 17 post initiation of differentiation using Direct-zol RNA Micro Prep Kit (Zymo Research) according to the manufacturer's directions. 500 ng of total RNA was used to make cDNA using qScript cDNA SuperMix kit (Quantabio). qPCR was performed in triplicate on 3 batches of differentiation (N=3, n=3) using iTaq Universal SYBR Green Supermix (Bio-Rad) on CFX Connect Real-Time System (Bio-Rad). Data points are represented as Log_2Fold Change relative to non-variable

conditions (i.e. No SHH, D4 SHH, or D4 SHH D8NGF). Statistical significance was determined by a one-sample t-test on Log_2Fold Change values.

Immunofluorescence

Resulting neurons were stained for markers using immunofluorescence at day ~45 post-induction of BFCN differentiation. Neurons were fixed with 4% paraformaldehyde (Electron Microscopy Sciences) for 15 minutes at room temperature and washed with 1X PBS. Non-specific binding sites were blocked, and cells were permeabilized for 15 min with 1X PBS containing 5% normal donkey (Jackson Immuno Research Laboratories Inc.) or goat serum (Jackson Immuno Research Laboratories Inc.), and 0.2% Triton X-100 (Acros Organics). Primary antibodies were applied at concentrations specified in table below in 1X PBS containing 5% normal donkey or goat serum and incubated overnight at 4°C. The following day samples were washed three times with 1X PBS. Secondary antibodies were incubated for 30 min at concentrations of 1:500 in 1X PBS containing 5% normal donkey or goat serum at room temperature. Successively, cells were washed once with 1X PBS. Lastly, the samples were stained with DAPI (Sigma) for 5 min and mounted with Vectashield (VectorLabs) on slides. Samples were stored in the dark at room temperature until imaging was performed on a Nikon Eclipse microscope.

Table 1. Primary Antibodies

Antibody	Host	Company	Cat. #	Concentration
B-Tubulin	Mouse	Abcam	32-2600	1:5000
ChAT	Goat	Millipore Sigma	AB144P	1:150

mCherry	Rabbit	Abcam	AB167453	1:1000
DAPI		Sigma		1:500

Discussion

Human pluripotent stem cells provide an excellent platform to generate neural populations, such as hPSC-derived BFCNs, and study their role in neurological disorders, such as Alzheimer's Disease and Down syndrome-related Alzheimer's Disease. However, few differentiation protocols have been established to derive BFCNs from hPSCs. Therefore, we took a systematic approach to target key developmental steps in the development of BFCNS by modulating the expression of NKX2.1, LHX8, and ISLET1.

BFCNs originate from the ventral regions of the telencephalon, and medial ganglionic eminences (MGE) (Sussel et al., 1999). MGE precursor cells that express LHX8 and ISLET1 differentiate to BFCNS *in vivo* (Bissonnette et al., 2011). Our protocol introduces SHH at day 4 which increased the MGE marker NKX2.1, NGF at day 8 to increase LHX8 expression, and a three-day pulse of BMP-9 at day 14 to enhance the expression of ISLET1.

Previously published BFCN protocols have shown an efficiency range between 30% – 90% (Hu et al., 2016, Muñoz et al., 2020, Bissonnette et al., 2011, Krajka et al., 2021, Ma et al., 2020). Many of the published BFCN protocols use direct reprogramming, sorting, or enrichment methods to generate a pure ChAT population. This makes it difficult to study the development of BFCNs. Bissonette et al. use similar small molecules, SHH and BMP-9, and utilize FACS sorting to enrich for LHX8 neural precursors to yield an efficiency of 85.59% ChAT positive neurons (Bissonnette et al., 2011). Ma et al. used two plasmids overexpressing key transcription factors, LHX8 and GBX1, to generate induced BFCNS resulting in an efficiency of 90% (Ma et

al., 2020). Protocols that do not use a filtering step tend to have lower efficiencies. For instance, Hu et al. differentiated iPSCs to BFCNs using SHH and NGF, resulting in an efficiency of 40% (Hu et al., 2016). We modulated the timing of the addition of key small molecules to result in an efficiency of 63%.

Our enhanced protocol does not use a selecting or sorting step and is more analogous to *in vivo* BFCN development, allowing us to study the development of BFCN *in vitro*. In addition to the ability to study early developmental cues, our model allows for long term culture and the investigation of potential mechanisms of vulnerability in diseases. BFCNs are vulnerable to degenerate Down syndrome- Alzheimer's Disease, however molecular pathways involved in BFCN degeneration are poorly understood. This system would be ideal to investigate mechanism leading to degeneration of BFCNS in individuals with Down syndrome.

Conclusion

In conclusion, our results identified that the addition of SHH on day 4, NGF on day 8, and BMP-9 pulse on day 14, was the most optimal combination to generate ChAT positive BFCNs from hPSCs. The significant increase in telencephalic ventral marker NKX2.1, along with BFCN progenitor markers LHX8 and ISLET1 after patterning reflects the regionalization of our human-derived cells into an MGE identity and eventually mature BFCNs reflecting the specified *in vivo* development of BFCNs *in vitro*.

Funding

This work was supported by the University of Wisconsin-Madison School of Medicine and Public Health (AB), and the Wisconsin Alumni Research Foundation (AB). JLM was supported by an R36 Dissertator Grant from the National Institute of Aging (R36AG072108), the

Advanced Opportunity Fellowship - SciMed Graduate Research Scholars at the University of Wisconsin-Madison, the University of Wisconsin Madison Biotechnology Training Program (NIH 5 T32 GM135066), and a fellowship from The Morse Society at the Waisman Center.

Acknowledgments

We thank Maddie Crockett, Kunal Damaraju, and Nicole West, for their valuable support and feedback.

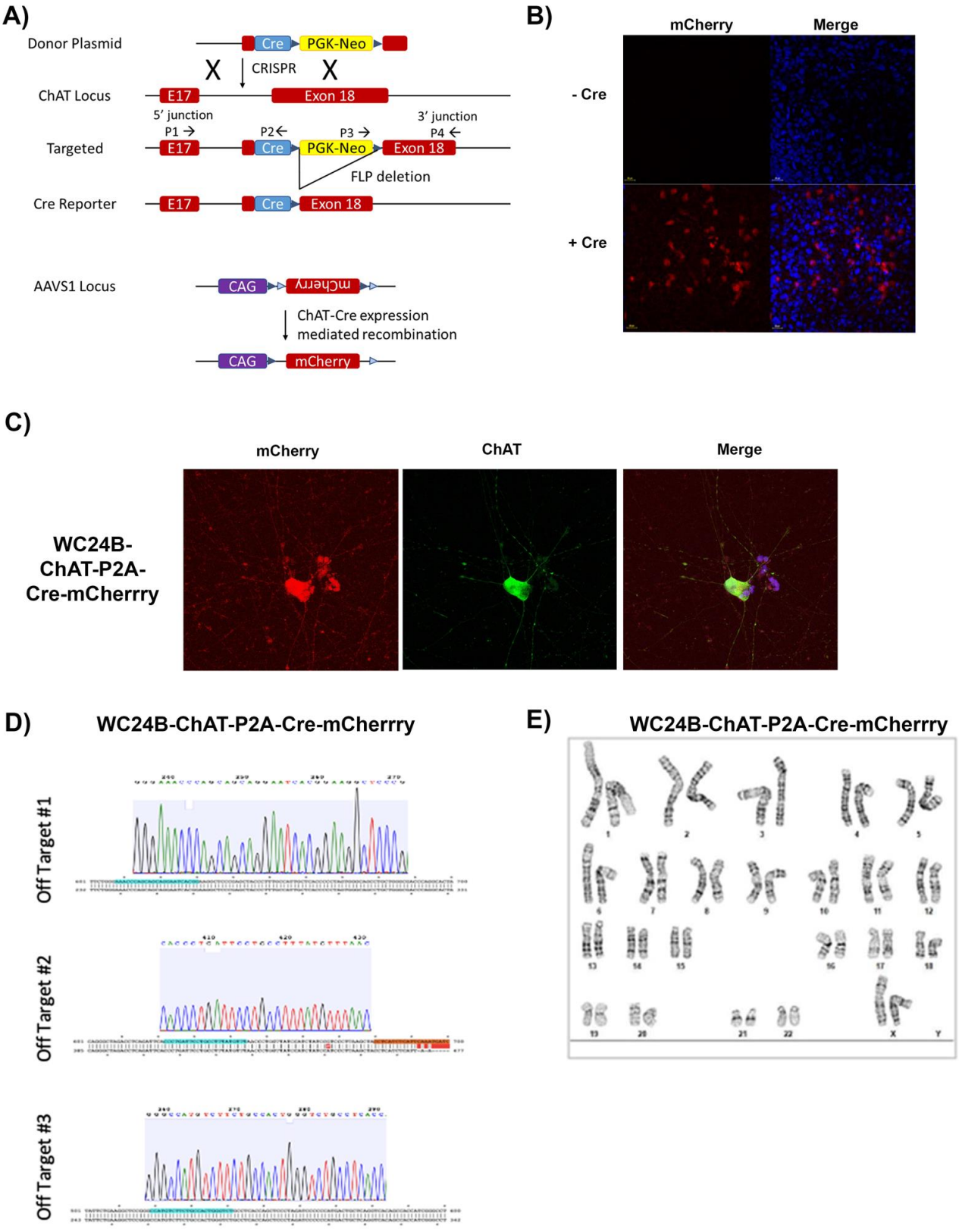

Figure S1. Generation of ChAT Reporter Line. A) Schematic of CRISPR/Cas-9 gene editing strategy. B-C) Immunofluorescence validation of CRISPR/Cas-9 gene editing via exogenous addition of Cas-9 and differentiation to BFCN. D) Off-target analysis validating the successful gene editing of iPSC. E) Karyotyping verification of iPSCs.

CHAPTER THREE: EXPLORING BASAL FOREBRAIN CHOLINERGIC NEURONS: AN EARLY HUMAN STEM CELL MODEL OF DOWN SYNDROME-ALZHEIMER'S DISEASE

Jose L. Martinez[1,5], Kunal Damaraju[5], Danielle Schmidt[5], Daifeng Wang[3,5], Andre Sousa[2,3], Anita Bhattacharyya[2,3*]

[1]Cellular and Molecular Biology Graduate Program, University of Wisconsin, Madison, Wisconsin

[2]Department of Neuroscience, School of Medicine and Public Health, University of Wisconsin, Madison, Wisconsin

[3]Department of Biostatistics and Medical Informatics, School of Medicine and Public Health, University of Wisconsin, Madison, Wisconsin

[4]Department of Cellular and Regenerative Biology, School of Medicine and Public Health, University of Wisconsin, Madison, Wisconsin

[5]Waisman Center, University of Wisconsin, Madison, Wisconsin

*** Correspondence:**

Anita Bhattacharyya, Ph.D.

bhattacharyy@waisman.wisc.edu

Abstract

Down syndrome (DS), or trisomy 21, is both a complex neurodevelopmental and neurodegenerative disorder. DS is characterized by altered cortical development resulting in Alzheimer's Disease (AD) pathology in middle age. It is estimated that more than 400,000 people are living with DS in the United States. Hallmarks of AD are present in all individuals with DS by mid-life. Additionally, cognitive functions decline through age as they develop AD pathology, with approximately a 90% lifetime risk of developing dementia. A critical feature that links cognitive impairment and neurodegeneration in trisomy 21 is the population of neurons that are susceptible in both processes: basal forebrain cholinergic neurons (BFCN).

BFCNs play key roles in regulating attention, memory, and learning. Degeneration or impairment of BFCNs leads to memory loss, decreased spatial recognition, and disturbance in language. Degeneration of this population has also been well documented in a diverse range of human neurocognitive disorders, including DS and AD. However, key molecular pathways involved in BFCN degeneration leading to cognitive, memory, and learning deficits aren't well understood. To fill a significant gap in the basic understanding of BFCNs, we explore the unique differences of BFCNs derived from both isogenic euploid and trisomy induced pluripotent stem cells. Single cell RNA sequencing was leveraged to better understand the molecular mechanisms that underlie the susceptibility of this subset of neurons. Results provide foundational data for identifying therapeutic targets for DS, as well as have an impact on our overall understanding of other neurodegenerative diseases such as AD.

Introduction

Down syndrome (DS, trisomy 21, T21) is a complex developmental disorder that is both a neurodevelopmental and a neurodegenerative condition and arises from trisomy of human

chromosome 21 (Lejeune, 1959). Due to medical interventions, significant improvements in the life expectancy of individuals with DS have been observed. The U.S. has seen an increase in the life span of people with DS of 456% between 1960 to 2007 (Presson et al., 2013). This increase in the life span of individuals with Down syndrome has resulted in systemic and age-related neurological conditions. In aging adults with DS, Alzheimer's Disease (AD) occurs at a higher prevalence and an earlier age than in typical aging adults.

Individuals with DS display the onset of neurodegeneration associated with Alzheimer's disease in early mid-life, with the prevalence of symptomatic AD in individuals with DS reaching 90 – 100% by age 70, while only 11% of the general population have AD by the age of 65 (2021, Fortea et al., 2020). While individuals with DS have significantly improved lifespans, these individuals have increased health complications with the development of dementia accounting for 70% of deaths in individuals over the age of 35 (Hithersay et al., 2019).

MRI data from the Down-Alzheimer Barcelona Neuroimaging (DABNI) and Sant Pau Initiative on Neurodegeneration (SPIN) study, identified a volumetric decrease of the basal forebrain with age of individuals with DS along the AD continuum (Rozalem Aranha et al., 2023). Additionally, cognitive decline associated in aging individuals with DS and AD is linked with the degeneration of the cholinergic basal forebrain system. This includes loss of BFCNs in the nucleus basalis and cholinergic fiber projections to the hippocampus and neocortex (Coyle et al., 1983, Coyle et al., 1988, Mufson et al., 1989b, Richter et al., 2022, Wegiel et al., 2022), identifying BFCNs as a critical neuronal population linking AD and DS.

The function of basal forebrain cholinergic neurons is crucial in mediating cognitive performance and memory throughout the lifespan, as evidenced by the correlation between memory loss in old age with a decline in the levels of acetylcholine, produced by BFCNs (White

and Ruske, 2002, Mori, 1997). Degeneration or impairment of BFCNs leads to memory loss, decreased spatial recognition, and disturbance in language. BFCN degeneration has also been well documented in a diverse range of human neurocognitive disorders including but not limited to Down syndrome, Alzheimer's Disease, and Parkinson's Disease. Administration of drugs that inhibit the breakdown of acetylcholine (e.g. donepezil) can reverse the memory deficits associated with aging and AD (Rusted, 1994, van Reekum et al., 1997). These data and others led to the "cholinergic hypothesis of AD" (Contestabile, 2011, Dumas and Newhouse, 2011, Coyle et al., 1983). However, key molecular pathways involved in BFCN degeneration leading to cognitive, memory, and learning deficits, and ultimately AD onset aren't well understood.

We set out to identify the potential mechanisms leading to the vulnerability of basal forebrain cholinergic neurons in the onset of Alzheimer's Disease in individuals with Down syndrome. To do so, we take advantage of isogenic iPSC models of Down syndrome to generate BFCNs and perform single cell RNA sequencing. Detailed comparisons of isogenic euploid to trisomy 21 BFCNs in our dataset reveal dysregulated pathways relating to apoptosis, mitochondrial metabolism, inflammation, and proteinopathies. Increased amyloid-β in trisomy 21 BFCN cultures suggests that this may be the first step in the cascade leading to their vulnerability in DS. We identify potential biological and molecular pathways that may lead to the degeneration of BFCNs, as well as provide a new human BFCN scRNAseq dataset that provides foundational data for identifying therapeutic targets for DS as well as enables future mechanistic studies on BFCN susceptibility in neurodegenerative diseases such as AD.

Materials and Methods

Differentiation of Basal Forebrain Cholinergic Neurons

Two pairs of isogenic trisomy 21 and euploid control iPSCs (Giffin-Rao et al., 2022, Weick et al., 2016), and one additional pair of trisomy 21 and euploid control iPSCs were used for this project (Table 1). iPSCs undergo regular karyotyping and mycoplasma testing to ensure quality (WiCell Institute, Madison WI). iPSCs were maintained on mouse embryonic fibroblasts (MEFs) feeder plates and differentiated to BFCNs using a protocol established in our lab (Fig. 1A).

Differentiation was initiated by detaching iPSC colonies with dispase to form aggregates known as embryoid bodies (EBs) at day 0. For the first 4 days, EBs were fed daily with hEB media (DMEM/F12, 20% knockout replacement serum, nonessential amino acids, L-glutamine, and β-mercaptoethanol) containing dual SMAD inhibitors (SB-431542 and LDN-193189 2HCl). Starting from day 4 until day 7, EBs were maintained in NIM (DMEM/F12, N2 supplement, nonessential amino acids, and heparin) containing dual SMAD inhibitors (SB-431542 and LDN-193189 2HCl) and SAG (Sigma Aldrich) and fed every other day for 3 days. EBs were attached to plates on day 7 using NIM containing dual SMAD inhibitors (SB-431542 and LDN-193189 2HCl), SAG, and 5% FBS for approximately 6 – 8 hours before replacing the media with NIM containing dual SMAD inhibitors (SB-431542 and LDN-193189 2HCl), and SAG. From day 9 until approximately day 14 plated EBs were fed every 2 – 3 days with NIM containing SAG and NGF (Invitrogen) until neural rosettes emerged. At this point, EBs are detached using a P1000 pipette and grown in suspension as neurospheres (NS), and maintained in NIM, SAG, and NGF media. For the first 3 days NS were given a pulse of BMP-9 (R&D Systems). Half of the NS media is replaced with fresh NIM every 3 – 4 days until day 25 when they are dissociated with Accutase (Sigma) and plated on coverslips coated with Poly-L-ornithine hydrobromide (Sigma) and laminin (Invitrogen) at a density of 25,000 – 30,000 cells per coverslip. Plated neurons are fed the following day with

500 uL of NDM (Neurobasal, N2, B27, Anti-anti, Glutamax, 30% glucose, GDNF (10 ng/mL), BDNF (10 ng/mL), Ascorbic Acid (200ng/mL), cAMP (1uM)) with CompoundE (Company), SAG and NGF, after which half media changes happen every 3 – 4 days with fresh NDM, SAG, and NGF until they are ready to analyze three weeks post-platting. Differentiations were performed in biological (N=3 pairs of euploid and trisomy 21 iPSC) and technical (n=3) triplicates.

Table 1. Information of iPSC Lines Utilized

iPSC Line	Condition	Gender/Age	Relationship
WC-24	Trisomy 21	Female/25y	Isogenic Pair 1
WC-24 U	Euploid	Female/25y	Isogenic Pair 1
DS1	Trisomy 21	Male/1y	Isogenic Pair 2
DS2U	Euploid	Male/1y	Isogenic Pair 2
WC20	Trisomy 21	Female/3y	Unrelated
WC58	Euploid	Female/Neonate	Unrelated

Immunofluorescence

BFCNs were assessed for markers using immunofluorescence at day ~45. Neurons were fixed with 4% paraformaldehyde (Electron Microscopy Sciences) for 15 minutes at room temperature and washed with 1X PBS. Non-specific binding sites were blocked, and cells were permeabilized for 15 minutes with 1X PBS containing 5% normal donkey (Jackson Immuno Research Laboratories Inc.) or goat serum (Jackson Immuno Research Laboratories Inc.), and 0.2% Triton X-100 (Acros Organics). Primary antibodies (Table 2) were applied in 1X PBS containing 5% normal donkey or goat serum and incubated overnight at 4°C. The following day samples were washed three times with 1X PBS. Secondary antibodies were incubated for 30 min

in 1X PBS containing 5% normal donkey or goat serum at room temperature. Successively, cells were washed once with 1X PBS. Lastly, the samples were stained with DAPI (Sigma) for 5 minutes and mounted with Vectashield (VectorLabs) on slides. Samples were stored in the dark at room temperature until imaging was performed on a STELLARIS-8 Leica Confocal Microscope or high-content imaging and analysis on the ImageXpress Nano Automated Imaging System. Experiments were performed in biological (N=3 pairs of euploid and trisomy 21 iPSC) and technical (n=3) triplicates.

Table 2. List of Antibodies

Antibody	Host	Company	Cat. #	Concentration
Map2	Chicken	Abcam	AB5392	1:5000
ChAT	Goat	Millipore Sigma	AB144P	1:150
P75NTR	Mouse	Advanced Targeting Systems	AB-N07	1:200
AT8	Mouse	Thermo Fischer Scientific	MN1020	1:500
HB9	Rabbit	Abcam	AB221884	1:300
H3K9ME3	Rabbit	Abcam	AB8898	1:2000
Casp3	Rabbit	Cell Signaling Technology	9661S	1:300
NeuN	Rabbit	Abcam	AB104225	1:1000
Islet1	Rabbit	Abcam	AB109517	1:200
vChAT	Rabbit	Synaptic Systems	139103	1:500
DAPI		Sigma		1:500

Single Cell Isolation

We generated a single-cell suspension by using the Jerber et al. protocol (Jerber et al., 2020). Cells were dissociated with Accutase (Sigma) with DPBS (Gibco) in a 1:1 ratio and incubated at 37 °C and 5% CO_2 with 0.5 mL of dissociation solution for approximately 20 minutes. Following incubation 2 mL of wash buffer was added (15mL DMEM:F12 + GlutaMAX (Gibco), and ROCK inhibitor (Y-27632, Tocris) to a final concentration of 10μM) to stop digestion and detached the cells from the plate. Cell suspension was transferred to a 15mL centrifuge tube capped with a 40μm filter and centrifuged at 150 x g for 3 minutes. The cells were washed and resuspended in wash buffer 2 (10% BSA solution (100 μg/mL) to DPBS (-/-) to a final concentration of 400 μg/mL). The resulting cell suspension was processed for cell number and viability analysis using Trypan Blue Solution (Sigma) and further diluted to the desired concentration for scRNA-seq. Isolation of single cells was performed in technical duplicates (N=1, n=2) with Isogenic Pair 1.

Single-Cell RNA Sequencing (10x Genomics)

All Single-Cell RNA Sequencing experiments were done on the Chromium Single-Cell platform (10x Genomics) (Fig. 2A). Cell barcoding, cDNA synthesis and the construction of the libraries were done using Chromium Next GEM Single Cell 3′ Kit v3.1 (10x Genomics, PN-1000269), Chromium Next GEM Chip G Single Cell Kit (10x Genomics, PN-1000127), and Dual Index Kit TT Set A (10x Genomics, PN-1000215) according to manufacturer's instructions. To generate single-cell Gel Bead-In-Emulsions (GEMs), isolated BFCN cell suspension was mixed with master mix, gel beads, and oil and then loaded on the Chip wells. Captured cells release RNA, which is reverse transcribed to synthesized full-length cDNA, which is amplified

to enough cDNA for library construction. The quality of cDNA was checked using Agilent Bioanalyzer. High-quality cDNA libraries were sequenced on the Illumina Novaseq X Plus platform by Novogene.

Single-Cell RNA Sequencing Analysis

Cell Ranger (10x Genomics) was used to process the Chromium Single-Cell RNA-seq output following established pipelines (Zheng et al., 2017, He et al., 2022). Raw base call files were demultiplexed in FASTQ files using "cellranger mkfastq" command. The FASTQ files were aligned to the 10x Genomics prebuilt reference Human genome (GRCh38.p13) to generate a data matrix using the "cellranger count" command. For quality control, cells with fewer than 500 or greater than 10,000 features and cells with higher than 10% mitochondrial contents were filtered out from the analysis.

Visualization and analysis were conducted using RStudio (RStudio v4.3.2, https://www.r-project.org), performed predominantly using Seurat v5.0.1 package(Hao et al., 2023). Analysis was performed in the following sequence, data normalization, log-transformation, highly variable genes selection and principal component analysis (PCA). Expression levels were normalized to counts per 10,000 using the Seurat function NormalizeData. Highly variable genes were selected based on the log-transformed data using the function FindVariableFeatures, the highly variable genes were visualized using VariableFeaturePlot, and the top 10 genes were labeled. Normalized Seurat object was integrated using IntegrateLayers before proceeding to the next steps. Integrated normalized Seurat object were scaled using ScaleData and PCA was performed with RunPCA. The heuristic elbow method was performed using ElbowPlot to determine the number of clusters in the data set. Nearest neighbors and Clustering were then

performed using FindNeighbors and FindClusters respectively. Uniform Manifold Approximation and Projection (UMAP) was generated using Seurat's RunUMAP function (Fig. 2B). Clusters of interest were identified using feature violin plots generated with VlnPlot function in Seurat (Fig. 2D-E). ISL1 (ISLET1), SLC18A3 (vChAT), and NTRK2 (TRKB) were uses as hallmark features of BFCNs (Fig. 2D). While MNX1 (HB9), MEIS2, SLC6A1 (GAT-1), were used as markers for motor neurons, lateral ganglionic eminence neurons (LGE), and GABA neurons respectively.

Differentially Expressed Genes (DEGs) for BFCN population were identified (Fig. 3A) by comparing Trisomy21 to Euploid BFCNs using the feature FindMarkers. DEGs with an adjusted p-value equal to or less than 0.05 (Fig. 3B) were used for Enrichr (http://amp.pharm.mssm.edu/Enrichr) analysis to perform a comprehensive Disease, GO-term, KEGG, and Pathway Analysis (Xie et al., 2021, Chen et al., 2013, Kuleshov et al., 2016).

Amyloid-β Concentration

Amyloid-β_{40} and amyloid-β_{42} concentrations were measured in our BFCN media. Spent media was collected at approximately day 45 post initiation of the differentiation to BFCNs. The Invitrogen Human Amyloid-β ELISA Kit (Human Aβ_{40} Cat. KHB3481, Human Aβ_{42} Cat. KHB3441) was used following manufacturer's instructions. Concentrations of Amyloid-β_{40} and Amyloid-β_{42} were measured with Molecular Devices VersaMax microplate reader. All samples were analyzed in biological (N=3 pairs of euploid and trisomy 21 iPSCs) and technical (n=3) triplicates.

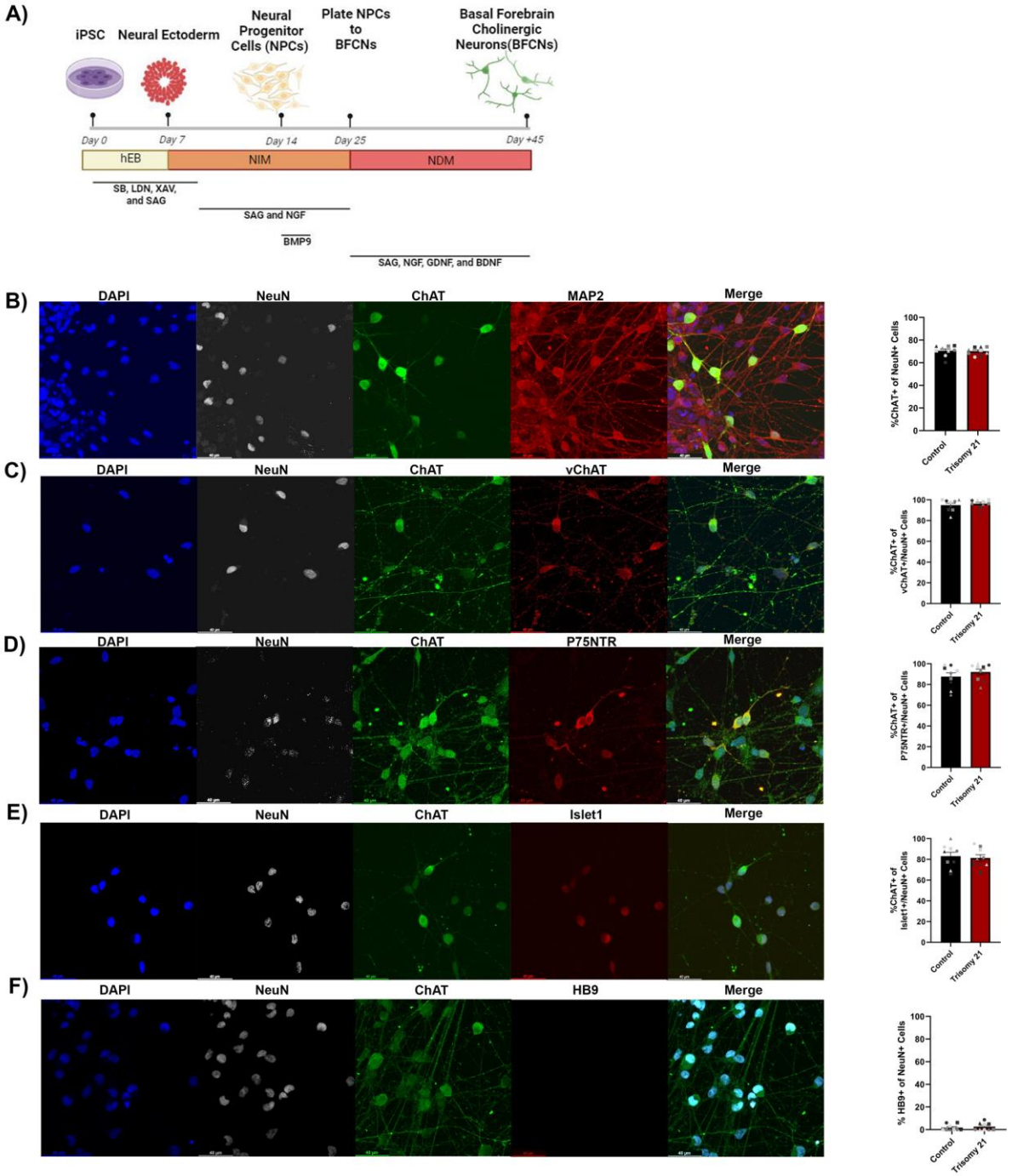

Figure 1. Generation of BFCN from Trisomy 21 and Isogenic Euploid iPSCs. A) Differentiation strategy to generate BFCNs. B-E) Quantification and immunofluorescence of BFCN markers; ChAT, vChAT, p75NTR, and Islet1. F) Quantification and immunofluorescence of HB9, a motor neuron marker. Statistical significance was determined by a one-sample t-test on Log2Fold Change values. Statistical significance was determined by a one-sample t-test.

Results

Validation of Basal Forebrain Cholinergic Neurons

To validate the differentiation of BFCN from isogenic euploid and trisomy 21 iPSCs, we used immunofluorescence to detect the presence of BFCN markers, ChAT, vChAT, ISLET1, and p75NTR, and the absence of motor neuron marker HB9. Choline acetyltransferase (ChAT) is key for the biosynthesis of acetylcholine, from acetyl-coenzyme A and choline (Cho et al., 2014), and is essential for the development of cholinergic interneurons. Thus, we assessed the presence of ChAT in our iPSC-derived BFCNs. Approximately 70% of NeuN positive cells also expressed ChAT, and no significant difference was observed between trisomy 21 and euploid BFCNs (Fig.1B). Cholinergic identity is also confirmed by the detection of the vesicular acetylcholine transporter (vChAT). Over 90% of ChAT expressing neurons co-expressed vChAT, with no significant difference observed between conditions (Fig.1C). In addition to the expression of ChAT, co-expression of p75NTR is additional evidence of mature BFCNS. Neurotrophin receptor p75 (p75NTR) encodes for a surface marker of mature BFCNs (Schnitzler et al., 2008). 90% of ChAT expressing neurons co-expressed p75NTR, with no significant difference between trisomy 21 and euploid BFCNs (Fig. 1D). ISLET1 is a ventral marker and is essential for the development of BFCNs (Furusho et al., 2006, Wang and Liu, 2001), which makes it a key validation marker for BFCNs. About 80% of ChAT-expressing neurons co-expressed ISLET1, with no significant difference between conditions (Fig. 1E). To confirm that the cholinergic neurons we produced are not motor neurons, we assessed the presence of key motor neuron marker HB9. Both euploid and trisomy 21 BFCN cultures showed negligible percentages (>5%) (Fig. 1F). The combination of these results suggests that our differentiation successfully generated BFCNs.

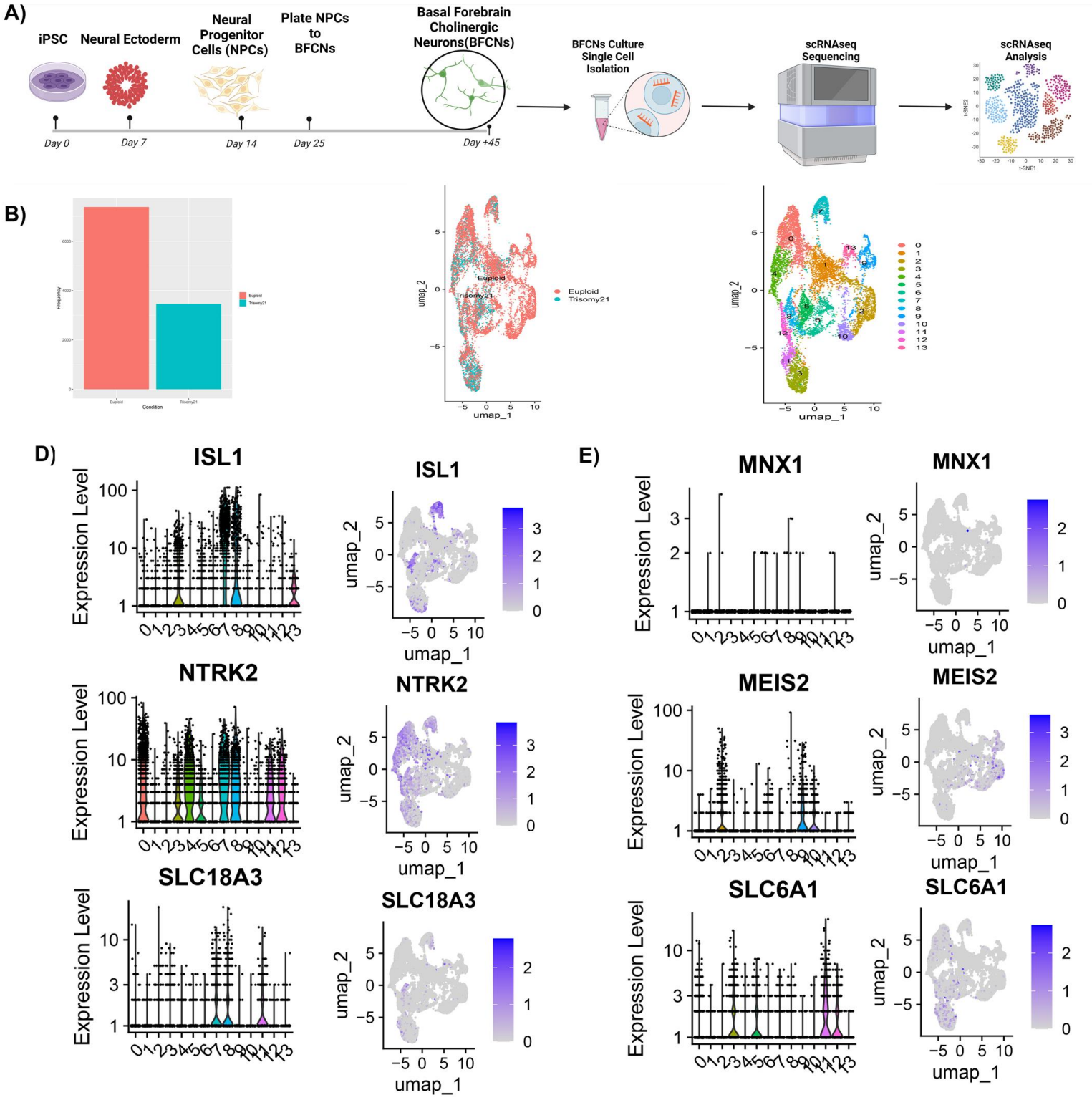

Figure 2. BFCN scRNAseq Analysis. A) Schematic of scRNAseq strategy. B) Quantification of the number of cells sequenced per condition. UMAP showing the single cell clustering identifying 13 unique clusters. C-D) Violin plot and UMAP feature graphs of cellular subtype markers.

Identification of scRNAseq BFCN clusters

Single cell RNA sequencing is a powerful tool for the detection and quantitative analysis of mRNA transcripts in individual cell types. To gain insight into the identity of the clusters resulting from our analysis, we performed manual annotation (Kim et al., 2023, Clarke et al., 2021) of scRNAseq data (Fig. 2A). Approximately 7,000 euploid and 3,500 trisomy 21 isogenic cells were analyzed to generate 13 clusters (Fig. 2B). Cluster analysis identified 13 unique cell clusters; using manual annotation clusters of interests, BFCNS were identified by considering their expression of at least four BFCN feature markers. Cluster 7 and cluster 8 are identified as BFCNs as they have high expression of ISL1 (ISLET1), SLC18A3 (vChAT), NTRK2 (TRKB), NTRK3 (TRKC), and ACHE (Fig. 2C, Fig. 1S), all of which are hallmark features of BFCNs. Cluster 3 also expressed of ISL1 and NTRK2, and ACHE; however, it did not express SLC18A3 which is a marker of mature BFCNs and thus it was excluded from DEG analysis (Fig. 2D, Fig. 1S). While hallmark genes of motor neurons (MNX1), LGE neurons (MEIS2), and GABA neurons (SLC6A1) are not expressed in clusters 7 and 8, cluster 3 did have expression of SLC6A1 (Fig. 2D). This expression data suggests that clusters 7 and 8 are BFCNs (Fig. 3A), while cluster 3 may be a subtype of BFCN.

Underrepresented Cell Populations in T21

Since twice as many euploid cells compared to T21 cells were sequenced and analyzed, we cannot make definitive statements about missing populations in the UMAP. However, cluster 2, cluster 9 and cluster 10 are underrepresented in T21 cells (Fig 3A). These cell populations all expressed MEIS2, which is a common marker for neuron populations that derive from the LGE (Schmitz et al., 2022). This data may suggest that T21 cell cultures undergoing our

differentiation protocol may respond to small molecules differently than their euploid counterparts, resulting in the depletion of LGE neurons.

Disease, Gene Ontology (GO), and Pathway Analysis

To gain insight into the impact of DEGs from BFCNs identified through single cell clustering (Fig. 3A), unbiased functional enrichment analysis was performed using Enrichr (Xie et al., 2021, Chen et al., 2013, Kuleshov et al., 2016). Thresholding for DEGs was established with an adjusted p-value <0.05 and Log2FoldChange >0 for upregulated and Log2FoldChange <0 downregulated genes (Fig. 3B). T21 is characterized by altered cortical development resulting in intellectual impairment at birth, as expected the enrichment analysis identified neurodevelopment disorders, intellectual disabilities, and Down syndrome in both the upregulated and downregulated gene sets (Fig. 3C-D). Interestingly neurodegenerative disorders and diseases, including Alzheimer's Disease, were also identified (Fig. 3C-D). This suggests that dysregulated genes in trisomy 21 BFCNs are strongly associated with pathways that may influence the early onset of AD in individuals with Down syndrome (Fortea et al., 2021).

Enrichment analysis of BFCN dysregulated genes identified key biological processes including apoptosis, inflammation, mitochondrial metabolism, and proteinopathies. Analysis of upregulated genes contained pathways related to positive regulation of apoptosis, negative regulation of metabolic process, and IL 24 signaling pathway (Fig. 3C). Enrichment analysis of downregulated genes contained pathways related to Mitochondrial Electron Transport, JAK/STAT signaling pathway, and Regulation of Tau-Protein Kinase Activity (Fig. 3D). Disruptions of key pathways leading to proteinopathies may predict faulty biological processes in T21 BFCNs resulting in their vulnerability ultimately leading to their degeneration and AD onset in DS.

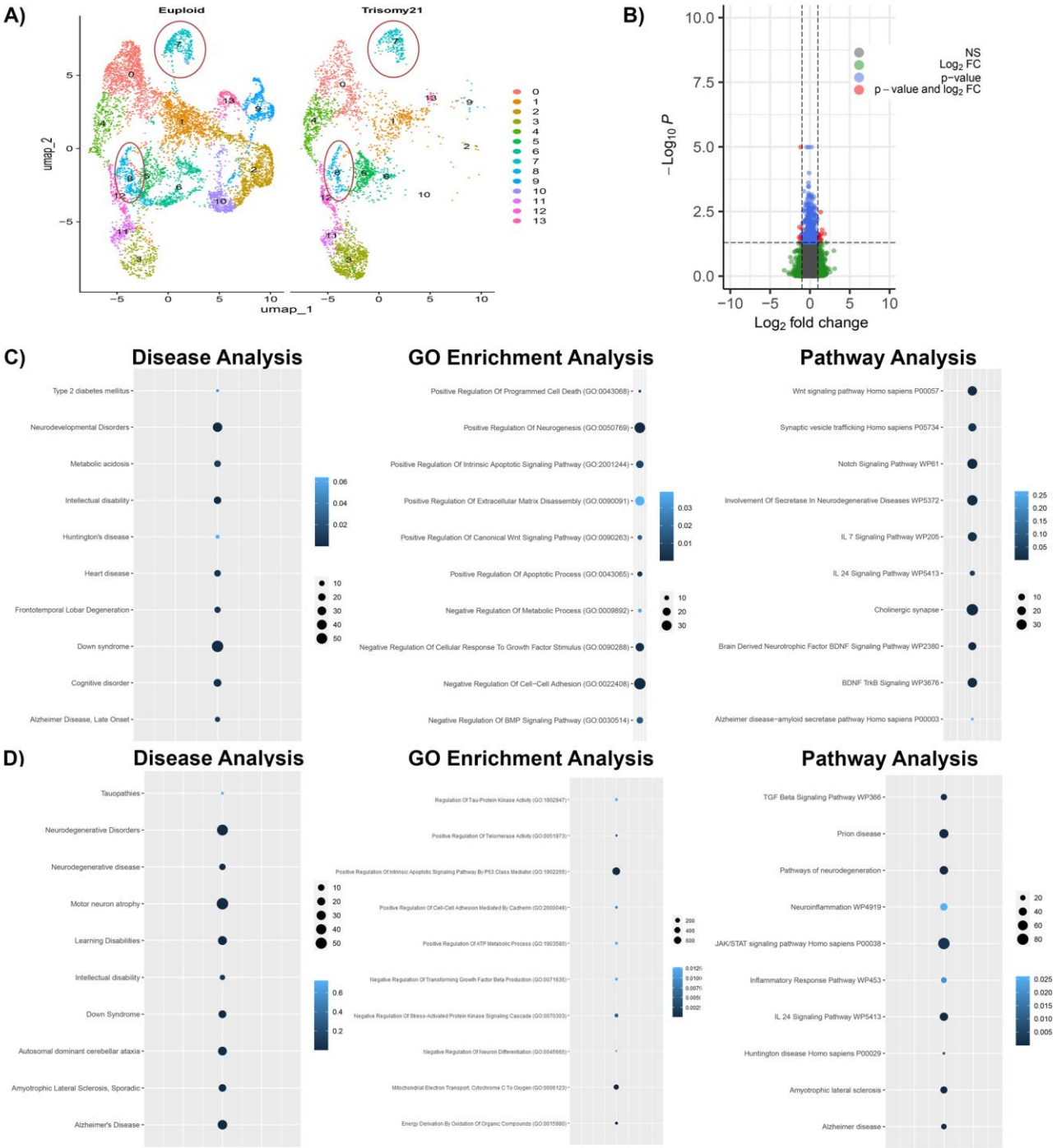

Figure 3. BFCN Enrichment Analysis. A) UMAP indicating clusters identified as BFCNs and used for DEG analysis. B) Volcano plot showing differentially expressed genes in T21 BFCNs. C-D) Bubble plots of Enrichment results highlighting Diseases, GO-Terms, and Pathway of up- and down- regulated genes.

Apoptosis and Proteinopathy in Trisomy 21 BFCNs

To further investigate the implications of key pathways identified in the enrichment analysis, we assessed neural apoptosis (Fig. 4A), phosphorylation of tau (Fig. 4C), and accumulation of amyloid-β (Fig. 4E) in our BFCN culture system. Upregulated genes in T21 BFCNs are implicated in the positive regulation of the intrinsic apoptotic signaling pathway, suggesting that this may be a cause of increased neural death in DS. This mechanism is activated by intracellular stress and ultimately results in permeabilization of the mitochondrial membrane via caspase activation resulting in cell death (Galluzzi et al., 2018, Green and Llambi, 2015). We assessed the abundance of the apoptosis effector Caspase 3 in our culture system (Fig. 4B). Although Caspase 3 was present in both euploid and T21 BFCNs, there was no significant difference in the average intensity observed. Suggesting that the intrinsic apoptotic signaling pathway may be indicating the activation of an initiator caspase rather than the effector caspase, Caspase 3.

Increased levels of soluble amyloid-β, the cleaved product of the amyloid precursor protein (Marks and Berg, 2003), and abnormal phosphorylation of tau (Swatton et al., 2004) both precede disease pathology in AD (Scheuner et al., 1996) and in individuals with Down syndrome (Tokuda et al., 1997). Unlike the general population, individuals with DS typically develop AD-like neuropathology as early as 30 (Wisniewski et al., 1985). Dysregulated genes in trisomy 21 BFCNs are associated with GO terms related to the regulation of tau-protein kinase activity. The associated gene set was downregulated in T21 BFCNs when compared to euploid, suggesting a change in the phosphorylation of tau. To assess the levels of phosphorylated tau, we calculated the average intensity of AT8 (pTau) in BFCNs (Fig. 4D). Results showed a slight increase in the average intensity in T21 but did not show any statistically significant difference.

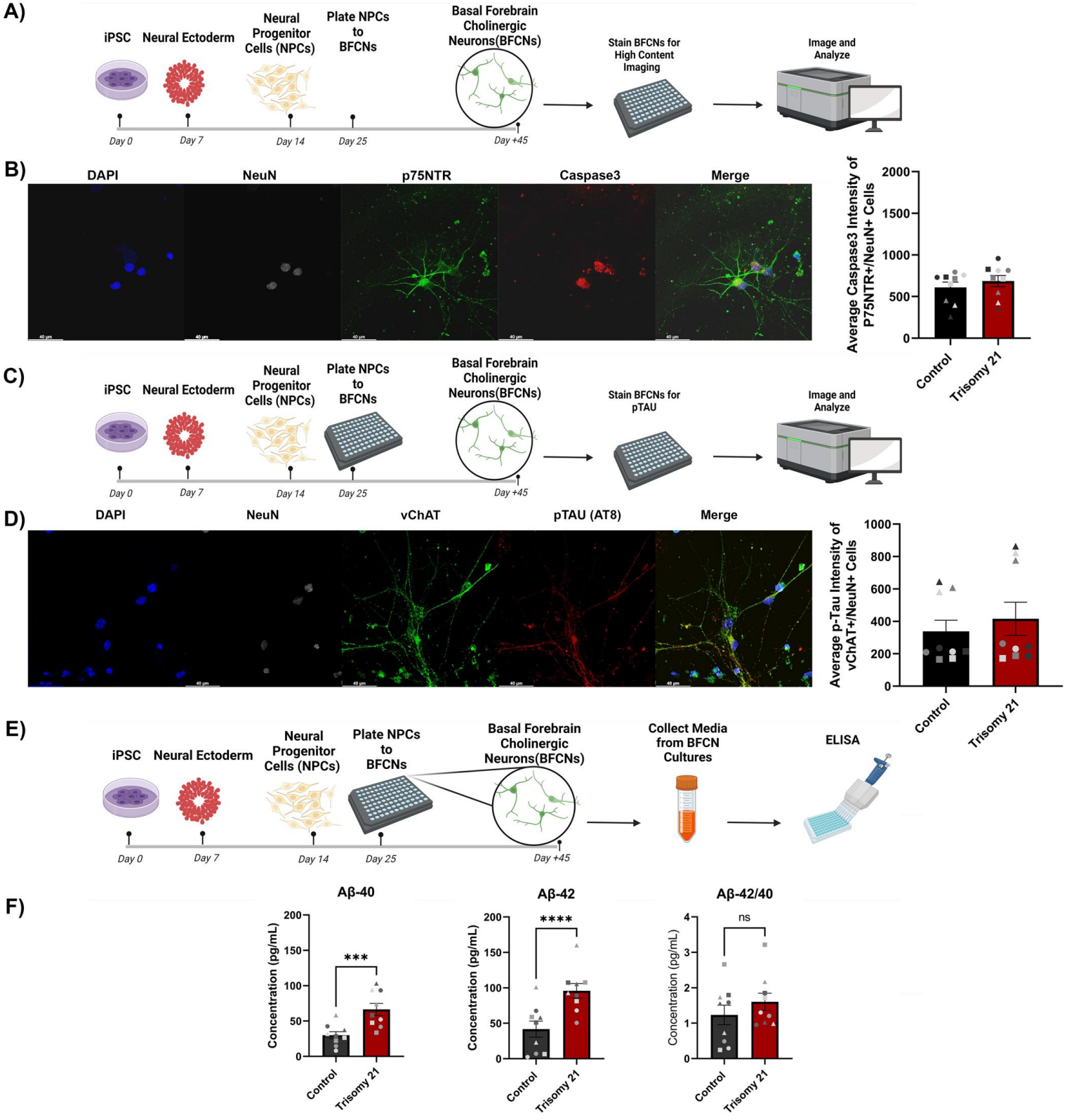

Figure 4. BFCN Pathway Validation. A-B) Experimental design, immunofluorescence, and quantification of Caspase3. C-D) Experimental design, immunofluorescence, and quantification of AT8. E-F) Experimental design and quantification of A concentrations. Statistical significance was determined by a one-sample t-test.

In addition to tau, amyloid-β is an associated protein in the neuropathy of AD. Increased differentially expressed genes in BFCNs were associated with Alzheimer disease-amyloid secretase pathway. ELISAs were performed to examine the levels of soluble cleaved product of the amyloid precursor protein in our BFCN cultures. Non-pathogenic $A\beta_{40}$ and pathogenic $A\beta_{42}$ were statistically significant as they were increased in trisomy 21 BFCN cultures as compared to euploid, however the $A\beta_{42}/A\beta_{40}$ ratio was not statistically significant (Fig. 4F). The $A\beta_{42}/A\beta_{40}$ ratio and the concentration of $A\beta_{42}$ on its own are biomarkers commonly used for diagnosis of AD (Hansson et al., 2019). When present in abnormally high levels, pathogenic $A\beta_{42}$ forms aggregates leading to plaques that collect between neurons and disrupt their function, resulting in AD (Oakley et al., 2006). Although we did not see a significant difference in the $A\beta_{42}/A\beta_{40}$ ratio in our culture system, this alone does not conclude that T21 BFCNs are not vulnerable to AD pathogenesis. This lack of significance can be explained by the statistically significant increase in $A\beta_{40}$, non-pathogenic, in cultured T21 BFCNs. The increased levels of pathogenic $A\beta_{42}$ in our culturing system suggests that T21 BFCNs may be susceptible to the onset of AD pathogenesis, as many have shown that the initial $A\beta_{42}$ aggregation forms independent of $A\beta_{40}$ (Gu and Guo, 2013).

Discussion

Diversity of BFCN subtypes

Single cell RNA sequencing allows for the profiling of thousands of cells in a single experiment. There are two main methods of annotation of resulting cell clusters: automatic cell annotation and manual cell annotation (Clarke et al., 2021). Automated cell annotation methods rely on curated marker gene databases, and reference expression datasets (Pasquini et al., 2021). Although automated cell annotation methods are convenient and systematic, there isn't a well-

established human BFCN reference database that can be used resulting in a low confidence in the ability to annotate clusters using this method. Manual annotation operates at the individual cluster level and relies on individual expert knowledge (Kim et al., 2023). This method is slower, labor intensive and it can be subjective, but offers a more precise labeling of clusters when there isn't a strong curated marker database that can be used for automated annotation. Using our own knowledge about BFCN development we annotated our scRNAseq clusters.

Our data identified three potential scRNAseq clusters of interest, two of which had at least four BFCN feature markers and one that only had three. This suggests the possibility of BFCN subtypes in our culturing system. First described in 1872, basal forebrain cholinergic neurons are a cluster of neurons in the basal forebrain (Meynert, 1872) organized into four categories. Ch1, originates from the medial septal nucleus, Ch2, from the ventricle limb of the diagonal band of Broca, Ch3, in the horizontal limb of the diagonal band of Broca, Ch4, developed in the basal magnocellular complex that includes the substantia innominate, the nucleus basalis of Meynert, the magnocellular preoptic nucleus and the ventral pallidum (Ballinger et al., 2016). BFCN structure and functions are regulated by neurotrophins binding to tropomyosin related kinase (Trk) receptors or p75 neurotrophin receptor (NTR). Nerve growth factor (NGF) binds to TrkA, brain-derived neurotrophic factor (BDNF) and NT-4 preferably bind to TrkB, while NT-3 binds to TrkC (Bothwell, 2014, Barbacid, 1994). Trk expression varies regionally, with the expression of both TrkB and TrkC being more prevalent in the nucleus basalis of Meynert (NbM), while TrkA is ubiquitous in other areas of the basal forebrain (Boskovic et al., 2019, Gibbs et al., 1989, Milne et al., 2015, Mufson et al., 1989a, Mufson et al., 2002, Salehi et al., 1996). Cluster 7 and cluster 8 both expressed TrkB, suggesting these are Ch4 BFCNs, belonging to the NbM. Cluster 8 also expressed TrkC, potentially making this an NbM BFCN subtype. Cluster 3 expressed TrkA, TrkB, and TrkC, but did not express vChAT, making

it a unique cluster and potential subtype of BFCN. This suggests that our culturing system allows us to generate at least three unique BFCN subtypes.

Pathways implicated in the vulnerability of BFCNs to AD

Due to the scarce availability of human BFCN data, we have relied on animal models to study BFCNs. Ts65Dn trisomic mouse model recapitulates both cognitive and morphological deficits of DS and AD, including the degeneration of BFCNs (Alldred et al., 2021, Alldred et al., 2023). Single population RNAseq of Ts65Dn BFCNs in the medial septal nucleus identified apoptosis signaling, mitochondrial dysfunction, and Alzheimer's disease amongst other dysregulated pathways in T21 (Alldred et al., 2021). This data supports our findings of dysregulated genes associated with cell death, mitochondrial metabolism, and proteinopathies.

The extensive neuronal loss that occurs in AD is speculated to be caused by the dysregulation of apoptotic death pathways and is etiologically responsible for the disease (Roth, 2001). We found that genes associated with the positive regulation of the intrinsic apoptotic signaling pathway were increased in T21 BFCNs. This finding suggests that we should observe an increase in cell death of BFCNs. Apoptosis can be divided into two intersecting pathways: intrinsic apoptosis and extrinsic apoptosis. Intrinsic apoptosis is activated by intracellular stress and results in permeabilization of the mitochondrial membrane through caspase activation (Galluzzi et al., 2018, Green and Llambi, 2015). Extrinsic apoptosis is caused by extracellular stimuli communicated through transmembrane death receptors and the formation of the death-inducing signaling complex (Fulda and Debatin, 2006, Galluzzi et al., 2018). We expected to see an increase in caspase 3 since this is an intrinsic pathway, yet our findings showed no significant difference. This can be due to the cell culture conditions that are designed to maintain optimal viability throughout the maintenance and neural differentiation of iPSCs.

Even though there were no significant differences in caspase, mitochondrial dysfunction pathways precipitated from the unbiased enrichment analysis. As a result of caspase activation, we anticipated increased mitochondrial membrane permeability resulting in the release of proteins that include caspase activators such as Cytochrome C (Galluzzi et al., 2009). Caspase 3 is an effector of apoptosis, which does not show early activation of the caspase pathway. Downregulated genes associated with mitochondrial electron transport from Cytochrome C to oxygen were observed, indicating that perhaps BFCNs are undergoing early activation of apoptosis through different caspase activators.

Amyloid-β is a well-established marker for the neuropathology of AD, with accumulation observed in individuals with DS as early as their 20's (Lott and Head, 2019, Lott et al., 2006). In response to increased amyloid-β in animal models of neurodegenerative diseases and in the AD brain, caspases have been implicated in the regulation of neural apoptosis (Roth, 2001). In T21 BFCN cultures there was a significant increase in the concentration of pathogenic amyloid-β, indicating that this might be a key initial step in the vulnerability of trisomy 21 BFCNs. As chromosome 21 includes amyloid precursor protein, we verified that this increase was not due to the intrinsic upregulation of the expression of APP. A Log2FoldChange of 0.17 was observed between euploid and trisomy 21, indicating that there is a negligible difference in the expression of APP. All this suggests that the increased levels of amyloid-β may elicit activation of caspases, which might be working to induce mitochondrial dysfunctions and ultimately lead to increased apoptosis of BFCNs in the NbM.

Conclusion

This comprehensive analysis generated 1) an iPSC-derived BFCN model of T21 and 2) the first single cell RNA seq data set of human BFCNs. Unbiased enrichment analysis confirmed that

the differentially expressed genes identified in T21 BFCNs are associated with neurodevelopmental and neurodegenerative diseases including Down syndrome, Tauopathies, and Alzheimer's Disease, as expected. Further analysis reveals that dysregulated genes impact pathways involved in apoptosis, metabolism, and proteinopathies. Ultimately, we identified a significant increase in amyloid-β_{42} suggesting that this may be a critical first step in the mechanisms of BFCN vulnerability to AD in individuals with Down syndrome.

Funding

This work was supported by the University of Wisconsin-Madison School of Medicine and Public Health (AB), and the Wisconsin Alumni Research Foundation (AB). JLM was supported by an R36 Dissertator Grant from the National Institute of Aging (R36AG072108), the Advanced Opportunity Fellowship - SciMed Graduate Research Scholars at the University of Wisconsin-Madison, the University of Wisconsin Madison Biotechnology Training Program (NIH 5 T32 GM135066), and a fellowship from The Morse Society at the Waisman Center.

Acknowledgments

We thank Karla M. Knobel for assistance with microscopy, Sara Knaack for assistance with single-cell RNA seq analysis, and Carissa Sirois, Nicole West, and Matt Russo for their valuable and insightful comments.

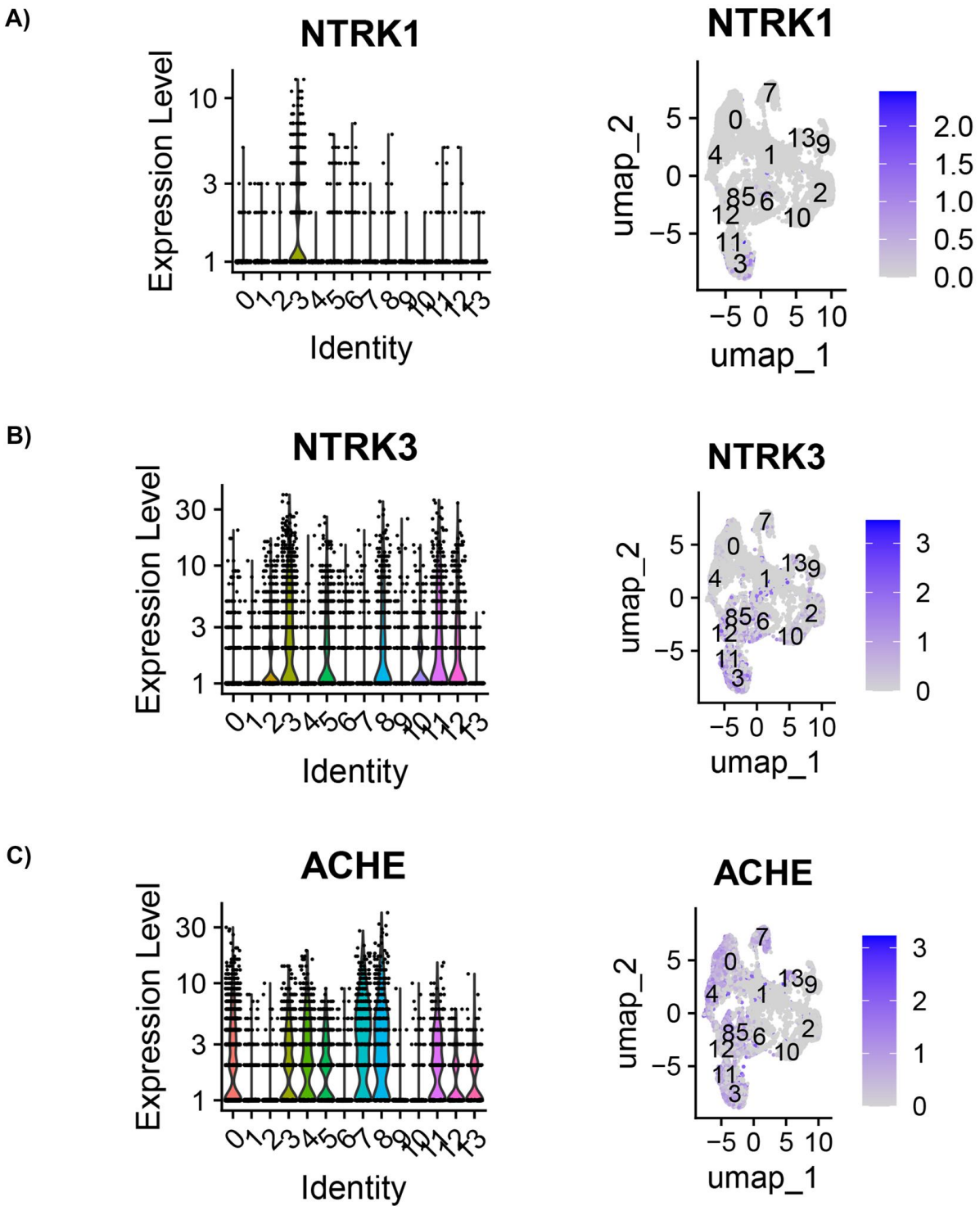

Figure 1S. BFCN Hallmark Markers. A-C) Violin plot and UMAP feature graphs of cellular markers, NTRK1 (TrkA), NTRK3 (TrkB), and ACHE (Acetylcholine) for BFCN subtype.

CHAPTER FOUR: LIMITATIONS AND FUTURE DIRECTIONS

Functional Analysis of Isogenic Euploid and T21 iPSC-derived BFCN

In Chapter Two we established a protocol for the generation of BFCNs from human-derived pluripotent stem cells relevant to trisomy 21 and their vulnerability to Alzheimer's Disease. To my knowledge, this is the first study to assess human trisomy 21 BFCNs. The validation of these iPSC-derived BFCNs was performed solely by immunofluorescence on three distinct iPSC pairs (N=3, n=3). This approach confirms the expression of key BFCN markers in our culture system such as CHAT, p75NTR, ISL1, and vChAT. Although immunofluorescence is a useful qualitative approach to identifying neurons of interest, ultimately neurons are defined by their functional properties which include electrical signaling.

This study lacked functional assays, such as electrophysiology, that would be useful to determine the maturity of the isogenic euploid and T21 iPSC-derived BFCNs. Thus, it is important that electrophysiology studies are performed, there are human BFCN electrophysiology datasets we can compare the results from these future functional assays. Whole-cell of iPSC-derived BFCNs by other groups have shown that *in vitro* 4-week-old BFCNs have a resting potential of approximately -32 mV, a cell capacitance of 21 pF, and can fire action potentials when the membrane potential is held at -60 mV (Bissonnette et al., 2011, Muñoz et al., 2020). In addition, murine ESC-derived BFCNs have a resting potential of approximately -30 mV (Yue et al., 2015). These datasets can serve as a reference to compare the results of our future electrophysiology studies.

BFCNs release acetylcholine and acetylcholine esterase in a healthy brain (Ballinger et al., 2016, Auld et al., 2001, Eickhoff et al., 2022). In addition to electrophysiology, acetylcholine and acetylcholine esterase ELISAs can be performed to further validate our mature BFCNs. ELISAs performed on spent media of 4-week-old murine ESC-derived BFCN contained 20 μM

acetylcholine and 50 mU/mL acetylcholine esterase (Yue et al., 2015). This murine dataset can serve as a reference to compare the results of our BFCN ELISA study.

Although extensive immunofluorescence was performed to validate BFCNs, further functional studies are needed. Electrophysiology studies of our iPSC-derived BFCNs would give us insight into their electrical activity. Acetylcholine and acetylcholine esterase ELISAs can further validate functional maturity of our iPSC-derived BFCNs. These additional studies would further validate our differentiation of mature and functional BFCNs.

Human BFCN scRNAseq Data Resource

Chapter Three resulted in a major resource for the scientific community, as we have generated the first human scRNAseq DS BFCN data set which enables human-specific studies at a single cell level. Up to this point, RNA sequencing studies of BFCNs in DS have predominantly been performed in animal models. The RNAseq data sets available are bulk RNA sequencing or are population enrichments through laser capture microdissections of murine BFCNs (Alldred et al., 2021, Jeong et al., 2016, Alldred et al., 2023). Although bulk RNA sequencing is informative, it is not 100% unique to neural subpopulation of interest, as it is typically performed on mixed neural cell cultures. scRNAseq allows us to perform gene expression analysis on the specific neurons of interest, generating BFCN specific data. Comparing our T21 BFCNs data to DS animal model bulk RNAseq data sets, similar pathways are affected including apoptosis, mitochondrial dysfunction, and proteinopathies, suggesting that some additional future work is necessary to further investigate these potential mechanisms of vulnerability.

There are limitations to our BFCN scRNAseq assay that we must consider and may require future work. Quality control of our sequenced single cells identified that approximately

half the number of cells were sequenced in trisomy 21 condition when compared to euploid (~3,500 trisomy and ~7,000 euploid). There are at least to possible explanations for this discrepancy: viability of T21 cells during the isolation of single cells, or at the time of sequencing resulting in more doublets in T21 than euploid, leading to the discarding of more cells in the T21 condition. Two simple approaches can be used to address this limitation. At the time of single-cell isolation count life-dead cells to see if one condition is more susceptible to cell death during this pre-sequencing steps. To address the lower number of trisomy 21 sequenced cells, sequence more iPSC-derived BFCNs to make sure a close to equal number of cells are analyzed. An additional limitation is that we performed all other assays in Chapter Three of this thesis as biological and technical triplicates (N=3, n=3), but our scRNAseq was not in biological replicates (N=1, n=2). To address this issue, additional biological replicates used in this thesis must be sequenced. The additional biological and technical replicates sequencing would address the limitations of this study.

Age-Relevant BFCN Models

Chapter Two resulted in an efficient protocol that does not use a selecting or sorting step and builds upon developmental principles of BFCN development *in vivo*. Although this strong model can be used to study the development of BFCNs and early-stage vulnerability to AD, it has its limitations. Reprogramming iPSCs from somatic cells erases aging markers found in donor cells (Kim et al., 2010), leading to hPSC-derived neurons that are similar to those found in fetal development (Lapasset et al., 2011, Menendez et al., 2011, Miller et al., 2013, Rando and Chang, 2012, Vera and Studer, 2015). The loss of these age-related markers found in the donor cells results in fetal signatures which require prolonged *in vitro* maturation to study diseases such as AD, making this a limitation of this study. To investigate effectors leading to BFCN

vulnerability to AD two approaches can be taken in the future to address this limitation; one is the use of "senescence cocktails" or "maturation cocktails" to enhance age-related phenotypes in neurons derived from iPSCs, and the second is the use of direct differentiation from donor cells (i.e. fibroblasts) of adult donors to generate BFCNs.

The use of pharmacological approaches to generate hPSC models that resemble adult and aging brains to model neurodegenerative diseases has been explored by others (Fathi et al., 2022, Hergenreder et al., 2022). SLO (SBI-0206965, Lopinavir, O151) is a "senescence cocktail" (Fathi et al., 2022) that has been shown to manipulate molecular markers of neuronal aging in iPSC-derived cortical neurons. After administration, SLO decreased expression of H3K9Me3, chromatin-associated protein HP1γ, and lamina-associated polypeptide Lap2β (Fathi et al., 2022). Suggesting that the introduction of this cocktail induces cellular senescence mimicking aging cells. GENtoniK (**G**SK-2879552, **E**PZ-5676, **N**MDA and Bay **K** 8644) a maturation cocktail, whose administration increased synaptic markers, Syn1 and PSD95, in addition to lowering AP thresholds and creating higher firing frequencies (Hergenreder et al., 2022). These results show that the maturation of hPSC-derived neurons can be enhanced by a simple pharmacological intervention, GENtoniK. Both pharmacological techniques can augment current modeling system to enhance the maturity of BFCNs derived from iPSCs and study AD-related phenotypes. Future work, a combination of these strategies with our BFCN protocol, would allow us to study the vulnerability of these neurons in different stages of maturity. The enhanced BFCN protocol allowed us to interrogate early neurons that are developing may be an initial step in the cascade leading to degeneration. The introduction of "senescence cocktail" or "maturation cocktail", would enhance the maturity of our BFCN cultures introducing the possibility of studying the mature age-like stage features of these neurons. Lastly, iPSC-derived BFCNs can be assessed at later time points than our original analysis, beyond 4 weeks, to study later-stage vulnerabilities

along the AD continuum in Down syndrome. However, this approach would not restore any of the age-related markers lost in the reprogramming of donor cells to iPSCs.

In addition to the cocktail strategy, a human-induced neuron approach would allow for the retention of aging markers and would be a more age-relevant model of BFCNs. Transdifferentiating fibroblasts or other aged somatic cells to BFCNs allows the complete avoidance of a pluripotent stage and maintains age-related markers (Mertens et al., 2015, Vierbuchen et al., 2010). Protocols have been established for the generation of aging-relevant BFCNs from adult human skin fibroblasts of sporadic AD patients (Ma et al., 2020). Human induced BFCNs (hiBFCNs) showed electrophysiological properties of mature neurons and BFCN markers, CHAT, p75NTR, ISL1, and vChAT. Future work using this hiBFCN approach to reprogram donor cells from individuals with DS would retain all age-related markers and features that may inform the vulnerability of BFCNs to AD. Further, identifying DS donor cells along the AD continuum (early pre-clinical, pre-clinical, MCI, and clinical AD) would allow us to identify key time points and biological processes leading to the vulnerability of BFCNs to AD by performing scRNAseq of hiBFCNs.

Oxidative Stress and Mitochondrial Deficits of iPSC-Derived BFCNs

Chapter Three generated a scRNAseq DS BFCN data set that identified biological processes that included oxidative stress and mitochondrial dysfunction. This thesis lacks studies of oxidative stress and mitochondrial dysfunction making it a limitation of this study. Some possible immediate next steps would be to investigate oxidative stress as it has long been implicated in DS (Lott et al., 2006, Perluigi and Butterfield, 2012, Murray et al., 2015b, Weick et al., 2013a). General cellular oxidative stress of DS iPSC-derived BFCNS can be performed using well-established protocols and commercially available kits such as CellROX (Invitrogen) or

DHE (Dihydroethidium, abcam). T21 hPSC derived dopaminergic neurons showed an elevated level of cellular oxidative stress when assayed with CellROX (Sun et al., 2022), while T21 iPSC derived cortical neurons exhibit increased oxidative stress using DHE (Weick et al., 2013a). The results of these experiments would validate scRNAseq results.

In addition to cellular stress, mitochondrial dysfunction was a key biological pathway that was disrupted in T21 BFCNs. Future work can be done to probe into mitochondrial membrane potential, mitochondrial oxidative stress, and mitochondrial metabolism. T21 iPSC-derived cortical neurons (Weick et al., 2013a) and T21 murine astrocytes (Shukkur et al., 2006) decrease in membrane potential when probed by JC-1 (new generation kit JC-10, abcam), indicating a decline in mitochondrial function. I would expect to see similar results in our BFCNs. Primary mixed cultures of DS human neurons and astrocytes have shown an increase in the presence of reactive oxygen species associated with mitochondrial dysfunction (Busciglio and Yankner, 1995). To show elevated reactive oxygen species in the mitochondria, leading to potential mitochondrial dysfunction in T21 iPSC-derived BFCNs, MitoSOX kit (Invitrogen) can be used. Fluorescent lifetime imaging microscopy (FLIM) has been used to probe mitochondrial metabolic processes by imaging naturally occurring NADH and FAD (Sanchez et al., 2018). Cortical T21 spheroids have been shown to have lower values of fluorescence lifetime contributions of the bound form of NADH indicating a higher reliance on glycolysis than mitochondrial respiration (Kashirina et al., 2021). These three assays would allow us to understand the impacts of oxidative stress on mitochondrial dysfunction in T21 iPSC-derived BFCNs.

In addition to these studies, it would be crucial to also understand neural morphology and localization of mitochondria as these inform neural health. T21 iPSC-derived GABAergic neurons had a less uniform distribution of mitochondria and had a more aggregative distribution

of mitochondria in neurons (Xu et al., 2022). In addition to this difference in mitochondrial distribution, T21 GABAergic neurons also decreased mitochondrial size and reduced mitochondrial metabolism. Future work needs to be done in DS iPSC-derived BFCNs to measure these mitochondrial metrics and neural morphology. The results would help us identify any morphological differences between euploid and T21 BFCNs, as well as identify the role of mitochondria on the vulnerability of BFCNs to T21 in individuals with DS.

Pathogenic Amyloid-β in T21 iPSC-Derived BFCNs

Increased pathogenic amyloid-β leads to the formation of amyloid-β plaques, a crucial step in the AD continuum. Amyloid-β is a well-established marker for the neuropathology of AD, with accumulation observed in individuals with DS in their mid-twenties (Lott and Head, 2019, Lott et al., 2006). In Chapter Three, T21 BFCN cultures showed a significant increase in the concentration of pathogenic amyloid-β, indicating that this might be an early step in trisomy 21 BFCNs. There is a negligible difference in the expression of APP between euploid and trisomy 21 BFCNs, suggesting that the increased levels of amyloid-β were not due to the intrinsic upregulation of the expression of APP. Perhaps, trisomy 21 intrinsic mechanisms of amyloid precursor protein cleavage led to the increase in pathogenic amyloid-β. AD iPSC-derived cortical organoids form amyloid-plaques validated by 4G8 and 6E10 (Penney et al., 2020). Future work is needed to assess if the increased concentration of amyloid-β identified in the ELISA leads to the increased formation of plaques in T21 BFCNs. It will be important to stain our iPSC-derived BFCNS with 4G8 and 6E10 to assess if there is a formation of amyloid-β plaques.

APPENDIX A: TRANSCRIPTIONAL CONSEQUENCES OF TRISOMY 21 ON NEURAL INDUCTION

Jose L. Martinez[1,2], Jennifer G. Piciw[1,2,3], Madeline Crockett[1], Isabella A. Sorci[1], Nikunj Makwana[1], Carissa L. Sirois[1], Yathindar Giffin-Rao[1], Anita Bhattacharyya[1, 4]

[1] Waisman Center, University of Wisconsin-Madison, Madison, Wisconsin, United States of America

[2] Cellular and Molecular Biology Graduate Program, University of Wisconsin-Madison, Madison, Wisconsin, United States of America

[3] Medical Scientist Training Program, School of Medicine and Public Health, University of Wisconsin-Madison, Madison, Wisconsin, United States of America

[4] Department of Cell and Regenerative Biology, School of Medicine and Public Health, University of Wisconsin-Madison, Madison, Wisconsin, United States of America

*** Correspondence:**

Anita Bhattacharyya, Ph.D.

bhattacharyy@waisman.wisc.edu

Preface

The text in this chapter was first published in the journal Frontiers in Cellular Neuroscience and was adapted for this thesis. The citation is as follows:

Martinez JL, Piciw J, Crockett M, Sorci I, Makwana N, Sirois C, Giffin-Rao Y, Bhattacharyya A. Transcriptional Consequences of Trisomy 21 on Neural Induction. Front Cell Neurosci. 2024 January 8. doi: 10.3389/fncel.2024.1341141.

Abstract

Down syndrome, caused by trisomy 21, is a complex developmental disorder associated with intellectual disability and reduced growth of multiple organs. Structural pathologies are present at birth, reflecting embryonic origins. A fundamental unanswered question is how an extra copy of human chromosome 21 contributes to organ-specific pathologies that characterize individuals with Down syndrome, and, relevant to the hallmark intellectual disability in Down syndrome, how does trisomy 21 affect neural development? We tested the hypothesis that trisomy 21 exerts effects on human neural development as early as neural induction. Bulk RNA sequencing was performed on isogenic trisomy 21 and euploid human induced pluripotent stem cells (iPSCs) at successive stages of neural induction: embryoid bodies at Day 6, early neuroectoderm at Day 10, and differentiated neuroectoderm at Day 17. Gene expression analysis revealed over 1,300 differentially expressed genes in trisomy 21 cells along the differentiation pathway compared to euploid controls. Less than 5% of the gene expression changes included upregulated chromosome 21 encoded genes at every timepoint. Genes involved in specific growth factor signaling pathways (WNT and Notch), metabolism (including interferon response and oxidative stress), and extracellular matrix were altered in trisomy 21 cells. Further analysis uncovered heterochronic expression of genes. This comprehensive analysis reveals that trisomy 21 impacts discrete developmental pathways at the earliest stages of neural development. Further, the results suggest that metabolic dysfunction arises early in embryogenesis in trisomy 21 and may thus affect development and function more broadly.

Introduction

The development of the nervous system begins with neural induction whereby pluripotent cells become restricted to a neuroectodermal fate. Neuroectoderm undergoes further development to generate neuroepithelial cells, or neural stem cells, which give rise to neural progenitor cells that will ultimately differentiate into neural cell types (neurons, astrocytes, oligodendrocytes). The ability to assess the early events of human neural induction is limited by the lack of accessibility to human embryonic tissue. *In vitro* culture of human pluripotent stem cells (PSCs) provides a means to analyze these early timepoints as neuroepithelial differentiation from human PSCs resembles *in vivo* neuroectoderm induction in its temporal course, morphogenesis, and biochemical changes (Pankratz et al., 2007, Lavaute et al., 2009, Kamiya et al., 2011, Huang et al., 2016).

Mistakes in any of the processes of neural development can result in neurodevelopmental disorders that are characterized by intellectual disability. In fact, genetic studies indicate that autism-related genes overlap with genes critical for all stages of neural development, including early neural induction (Casanova and Casanova, 2014), reflecting the importance of fidelity in these processes. The most common genetic cause of intellectual disability is Down syndrome (DS) caused by trisomy 21 (T21). Defects in neurogenesis and reduced cortical size have been established in mid-late gestation fetuses and neonates with T21, indicating errors in prenatal neural development (Wisniewski et al., 1984, Schmidt-Sidor et al., 1990, Guidi et al., 2008, Guidi et al., 2011, Guidi et al., 2018, Stagni et al., 2019, Patkee et al., 2020, Baburamani et al., 2020, Tarui et al., 2020, Golden and Hyman, 1994, Ross et al., 1984, McCann et al., 2021, Guihard-Costa et al., 2006). Yet, these results represent endpoints of neural development, and there is little to no information about the impact of T21 on the earliest stages of nervous system formation.

Induced PSCs (iPSCs) generated from individuals with DS enable modeling of neural development in a disorder whose genetic basis is not easily reproduced in animal models (Antonarakis, 2001, Hibaoui et al., 2014, Gardiner and Davisson, 2000, Sturgeon and Gardiner, 2011). Here, we use this powerful cellular paradigm to address a key gap in understanding the impact of T21 on early neural development. Using T21 and isogenic euploid control iPSCs, we interrogated the molecular impact of T21 on neural induction using bulk RNA sequencing. Our results reveal that T21 dysregulates WNT signaling and increases inflammatory response and oxidative stress, highlighting the impact of T21 on initial stages of neural development.

Materials and Methods

Cell culture and differentiation

Isogenic pairs of trisomy 21 and euploid control iPSCs were generated from fibroblasts mosaic for trisomy 21(Giffin-Rao et al., 2022, Weick et al., 2016). iPSCs undergo regular karyotyping and mycoplasma testing to ensure quality (WiCell Institute, Madison WI). iPSCs were maintained on mouse embryonic fibroblasts (MEFs) feeder plates and differentiated to definitive neuroectoderm following previously published studies (Chambers et al., 2009, Zhang et al., 2001, Pankratz et al., 2007). Briefly, iPSC differentiation was initiated by detaching iPSC colonies with dispase to form aggregates (embryoid bodies (EBs)) at day 0. EBs were fed daily with hEB media (DMEM/F12, 20% knockout replacement serum, nonessential amino acids, L-glutamine, and β-mercaptoethanol) with dual SMAD inhibitors (SB-431542 and LDN-193189 2HCl) for 4 days. From days 4-7, EBs were maintained in neural induction media (NIM) (DMEM/F12, N2 supplement, nonessential amino acids, and heparin) and fed every other day for 3 days. At day 7, EBs were allowed to attach to plates using NIM and 5% FBS for approximately 6 – 8 hours before the media was changed to NIM. Plated EBs were fed every 2-3 days with NIM until neural rosettes,

indicative of neural stem cells, emerged (~Day 14) when they are detached and grown in suspension as neurospheres (NS) and maintained in NIM media. Three technical replicates (batches of differentiation) were carried out for each experiment.

Total RNA Isolation and Bulk RNA sequencing

Total RNA was isolated from all timepoints using Direct-zol RNA Micro Prep Kit (Zymo Research) according to manufacturer's directions. Samples were eluted with nuclease free water and validated for quality based on 260:280 values and concentration. RNA sample quality, preparation of a 1 x 100 bp stranded mRNA poly-A tail enriched unpaired end library preparation and RNA sequencing was performed by the Biotechnology Center at the University of Wisconsin – Madison on the Illumina HiSeq 4000 platform.

Data Analysis

Bulk RNA-seq resulting reads were aligned to the human genome (GRCh37/hg19) and gene counts extracted using the Spliced Transcripts Alignment to a Reference (STAR v2.7.11a) package (Dobin et al., 2013). Count files were transferred to RStudio (RStudio v3.5.3, https://www.r-project.org) and merged into a single table per experimental timepoint. Zero count genes and the bottom 10% of genes with counts were removed. Counts were normalized by size factors and differentially expressed genes (DEGs) analysis was done using DESeq2 v1.22.2 (Love et al., 2014). DEGs were obtained by comparing the results of the isogenic control cells against the results of the trisomy 21 cells. Principal Component Analysis was performed on normalized counts using plotPCA in ggplot2 v3.3.5 package in RStudio. Thresholding for DEGs was established with an adjusted p-value <0.05 and Log2FoldChange >1.0. The RStudio package

pheatmap v1.0.12 was used to plot the heatmaps and hierarchical clustering was done using the distance matrix with Pearson's correlation. Volcano plots were generated with the Enhancedvolcano v1.13.2 package in RStudio to visualize significant DEGs. The top ten DEGs based on adjusted p-value were highlighted in the plots. All Venn diagrams were generated with the ggvenn v0.1.10 package in RStudio. Gene expression profiles of our identified clusters were generated using the ggplot2 v3.3.5 package in RStudio implementing a linear model method.

GO-Term and Pathway Analysis

Differentially expressed genes were filtered by adjusted p-value<0.05 and a log2FoldChange>1 using RStudio. Further filtration was done by removing genes that did not have a count greater than zero throughout the entirety of our differentiation timepoints. The resulting 96 genes were further divided into 4 gene lists that represent the identified expression profile clusters in our heatmap hierarchical clustering analysis. Enrichr (http://amp.pharm.mssm.edu/Enrichr) was used to perform a comprehensive GO-term, KEGG, and Pathway Analysis (Xie et al., 2021, Chen et al., 2013, Kuleshov et al., 2016). Separate Enrichr analyses were performed for each of the identified cluster gene list.

qPCR

Total RNA was isolated from three replicates of differentiation for all timepoints using Direct-zol RNA Micro Prep Kit (Zymo Research) according to manufacturer's directions. 500 ng of total RNA was used to make cDNA using qScript cDNA SuperMix kit (Quantabio). qPCR was performed in triplicate on 3 batches of differentiation (N=3, n=3) using iTaq Universal SYBR Green Supermix (Bio-Rad) on CFX Connect Real-Time System (Bio-Rad). Data are presented as

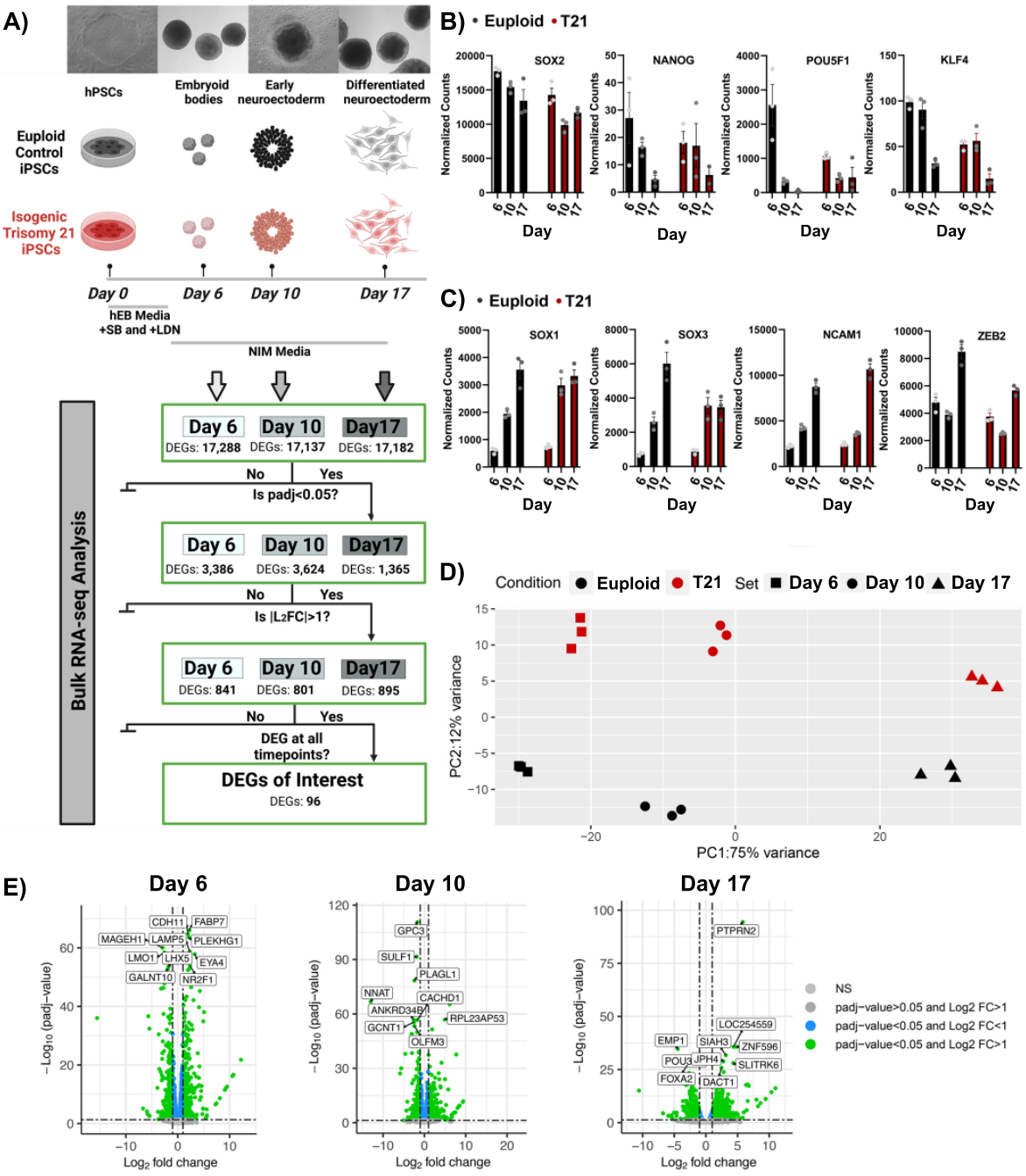

Figure 1. Transcriptional differences between isogenic Trisomy 21 and euploid iPSCs during neural induction. A) Experimental design showing differentiation strategy from a pair of control and isogenic T21 human iPSCs (WC-24-02) differentiated to dorsal forebrain neural progenitor cells and analyzed by RNA

ddCt values. Error bars indicate ddCt values ± 1 SD. Statistical significance was determined by one-sample t test on ddCt values.

Results

Identification of transcriptional differences between isogenic Trisomy 21 and control iPSCs during neural induction.

To identify transcriptional consequences of T21 during neural induction, a pair of control and isogenic T21 human iPSCs (WC-24-02) were differentiated to dorsal forebrain neural progenitor cells (Fig. 1A). To validate our neural differentiation, we assessed the expression of pluripotency genes SOX2, NANOG, POU5F1, and KLF4. As expected, pluripotency gene expression was downregulated in both euploid and T21 cells as they differentiated (Fig. 1B). Concurrently, the expression of neuroectoderm genes SOX1, SOX3, NCAM1, and ZEB2 were upregulated as expected in both euploid and T21 cells as they differentiated (Fig. 1C). The downregulation of pluripotent genes and upregulation of neuroectoderm genes confirmed the conversion from iPSCs to neuroepithelium in culture (Huang et al., 2016).

Bulk RNA sequencing of samples was performed at three timepoints: day 6 embryoid bodies, day 10 early neuroectoderm and day 17 differentiated neuroectoderm (Fig. 1A). Principal component analysis (PCA) of gene counts revealed that the samples segregate by stage along neural induction (75% variance) and cluster by disorder (T21 versus control) at each timepoint (12% variance) (Fig. 1D). Heatmap hierarchical clustering of samples by transcriptomic data shows that samples segregated into control and T21 at each individual timepoint (Fig. S1A-C). These results indicate that gene expression changes driven by T21 can be detected during the process of neural induction. Differentially expressed genes (DEGs) in T21 versus control were identified at each timepoint (Fig. 1A). DEG analysis and hierarchical clustering reveal significant

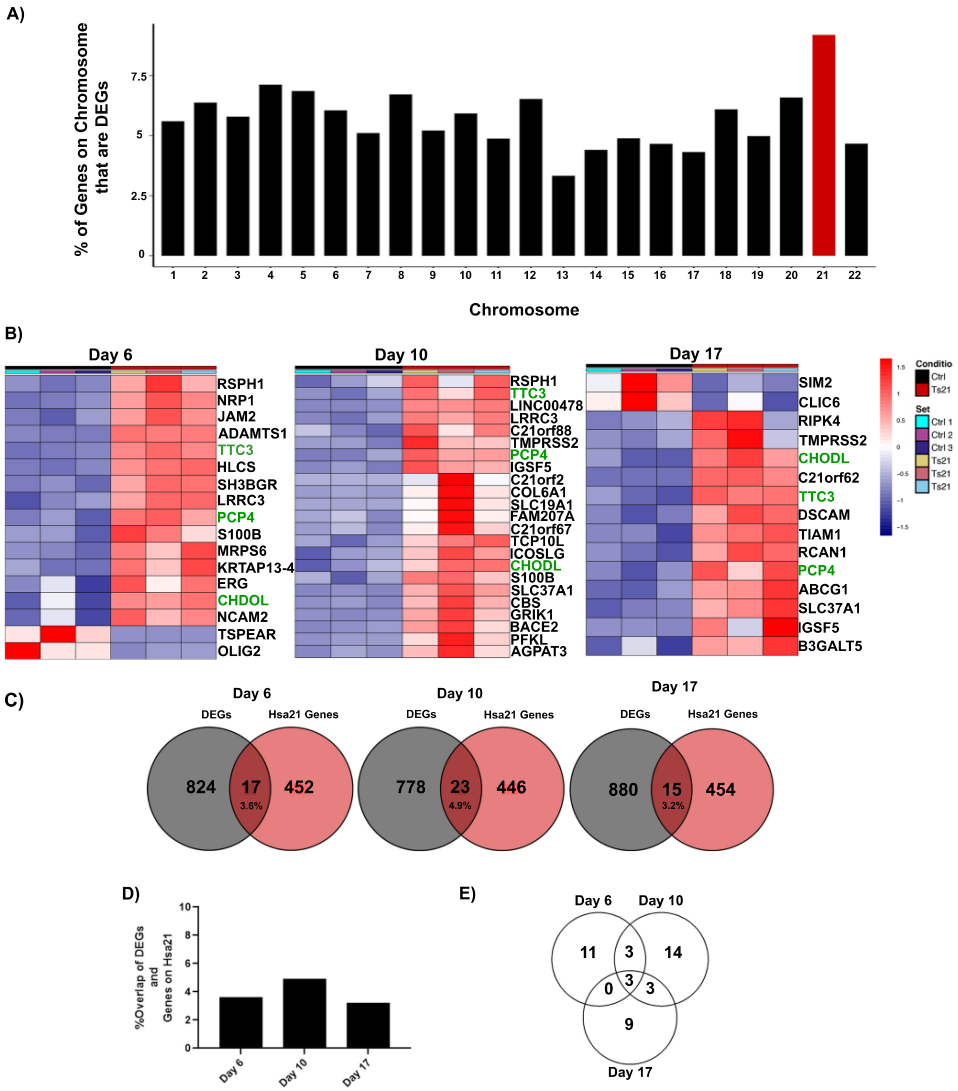

Figure 2. Differential gene expression of HSA21 encoded genes. A) Graph representing the percent of genes per chromosome that are significant DEGs. B) Heatmap of differentially expressed chromosome 21 genes using DEGs with a padj<0.05 and L2FC>1. Time course replicates are labeled with light gray for day 6, medium gray day 10, and dark gray day 17. Isogenic Control is highlighted by black and T21 by red. Upregulated genes are displayed in red while downregulated genes are expressed in blue, genes that are present at all timepoints are highlighted in green text. C) Venn diagram showing the number of chromosome 21 genes overlapping throughout the timepoints. D) Venn diagram showing the number of chromosome 21 genes overlapping with DEGs that have a padj <0.05 and L2CF>1. Number of unique chromosome 21 genes in red and number of non-overlapping DEGs in gray, overlapping area shows percentage overlap. Graph showing percent overlap at each timepoint.

differences in expression profiles between T21 and control at each stage: Day 6 / EBs (17,288 genes), Day 10 / early neuroectoderm (17,137 genes) and Day 17 / differentiated (17,182 genes; Figure 1E; Fig. S1A-C). Using stringent cutoffs of adjusted p-value (padj<0.05) and log2FoldChange (|L_2FC|>1), we identified 841 DEGs at day 6, 801 DEGs at day 10, and 895 DEGs at day 17 (Fig. 1A, Fig. 1E). 96 DEGs are consistently dysregulated throughout the time course (Fig. 1A) suggesting that they play a continuous role in neural induction in the context of T21.

Few differentially expressed genes are encoded by HSA21

Genes encoded by human chromosome 21 (HSA21) are expected to have increased expression because of their higher gene dosage. To assess the relative contribution of HSA21 genes, we sorted the results of our differential gene expression analysis by chromosome. Genes encoded in all chromosomes were differentially expressed (Fig. 2A). As expected, HSA21 has the highest percentage of DEGs based on the proportion of genes on each individual chromosome (Fig. 2A). Only 17 HSA21 genes at day 6, 23 HSA21 genes at day 10, and 15 HSA21 genes at day 17 meet our criteria (Fig. 2B-C). Although these HSA21-encoded DEGs are generally overexpressed, TSPEAR and OLIG2 at day 6, and SIM2 and CLIC6 at day 10 were down-regulated (green in Fig. 2B). HSA21 DEGs represented 3.6% of all DEGs at day 6, 4.9% of all DEGs at day 10, and 3.2% of all DEGs at day 17 (Fig. 2C, D). Only 3 HSA21 genes (PCP4, TTC3, and CHODL) were expressed throughout the differentiation (Fig. 2E). The low percentage of dysregulated HSA21 genes indicates that T21 has broad transcriptional consequences across the genome early in neural induction.

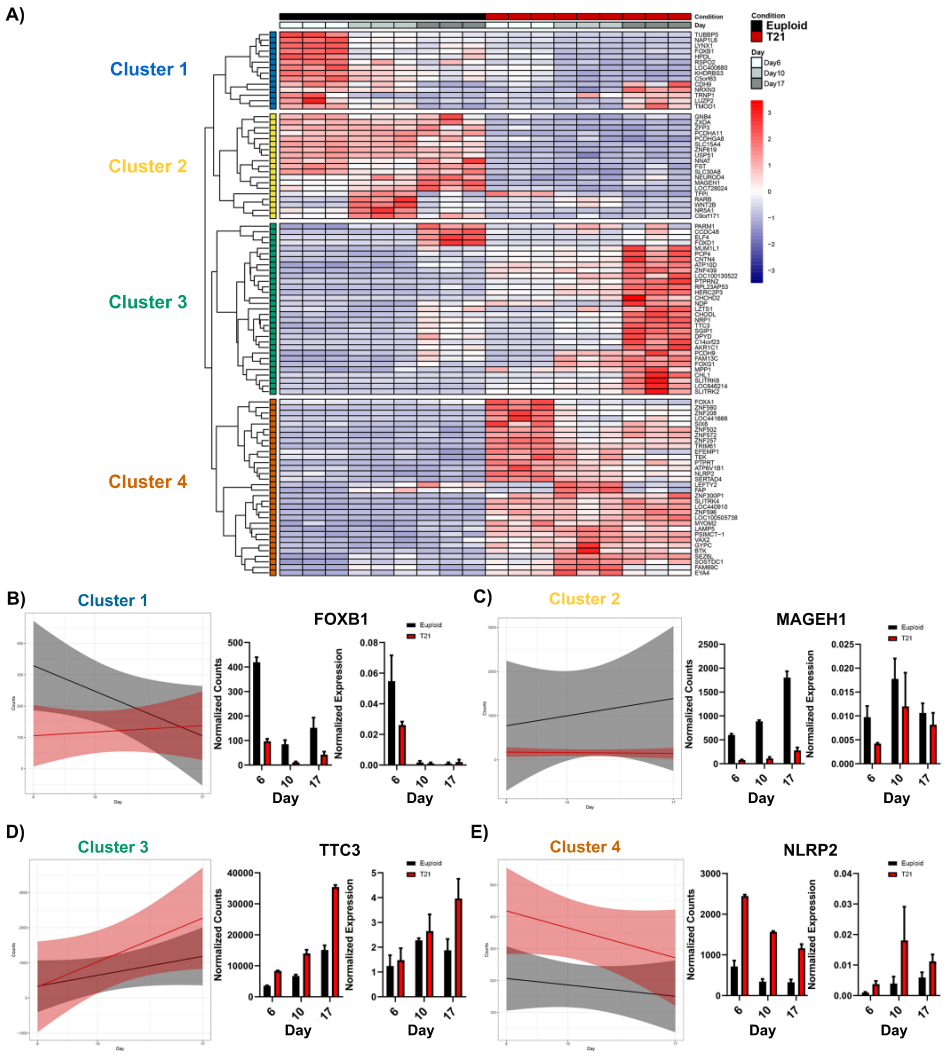

Figure 3. Transcriptional Changes in T21 and Isogenic Control Identified Four Major Expression Patterns. A) Heatmap of differentially expressed genes using DEGs with a padj<0.05 and L2FC>1. Time course replicates are labeled with light gray for day 6, medium gray day 10, and dark gray day 17. Isogenic Control is highlighted by black and T21 by red. Upregulated genes are displayed in red while downregulated genes are expressed in blue. B, C, D, E) Plot showing linear model of the average expression trends of DEGs in each cluster over the timecourse of differentiation to neuroectoderm. Graphs showing a representative gene expression trend over the timecourse. On the left are normalized bulk RNAseq counts and on the right is normalized expression of the gene using quantitative PCR (N=3 batches, n=3 technical replicates). Error bars indicate ddCt values ± 1 SD. Statistical significance was determined by one-sample t test on ddCt values, no significant difference was found.

Genes with constant expression and dynamic expression patterns reveal four unique clusters.

To explore the transcriptional impact of T21 throughout neural induction, we performed unbiased hierarchical clustering of our 96 DEGs to identify patterns of altered regulation. Results reveal four clusters with unique and dynamic expression profiles, identified through linear model analysis (Fig. 3A). Cluster 1 genes are expressed only at day 6 in euploid cells, while the genes have low or no expression in T21 cells (Fig. 3A, B). Cluster 2 identified genes that are expressed in euploid cells and down-regulated in T21 cells throughout differentiation (Fig. 3A, C). Cluster 3 revealed a heterochronic pattern of gene expression in which the genes are highly expressed earlier in T21 cells compared to euploid cells (Fig. 3A, D). The last cluster, Cluster 4, represents genes that were upregulated in T21 cells compared to euploid cells at all differentiation timepoints (Fig. 3A, E). The expression pattern of one gene from each cluster was validated by qPCR (Fig. 3 B-E). These distinctive expression patterns indicate that each cluster has a unique impact driven by T21 during neural induction.

Gene Ontology (GO) and Pathway Analysis

To gain insight into the impact of the gene expression patterns revealed by hierarchical clustering, unbiased functional enrichment analysis was performed using Enrichr (Xie et al., 2021, Chen et al., 2013, Kuleshov et al., 2016) to identify molecular pathways and biological processes that are represented by each cluster. Enrichment analysis identified key biological processes including regulation of cell death, immune response, inflammation response, metabolism, and extracellular matrix that are impacted by T21 during neural differentiation. Cluster 1 contained genes in pathways related to brain development and regulation of cell death, which was anticipated given that our experimental paradigm is neural development (Fig. 4A). Decreased expression of

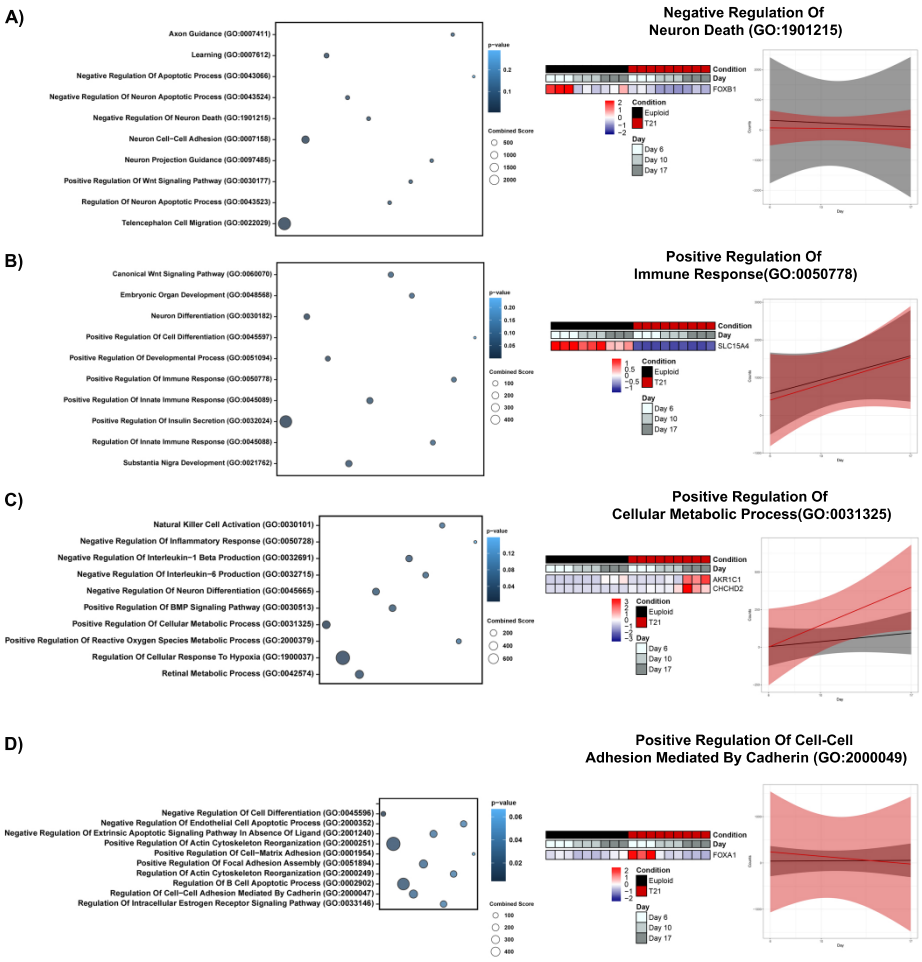

Figure 4. GO Analysis of Identified Four Major Expression Patterns. A) Bubble plot highlighting unique GO Terms to Cluster 1, relating to brain development and regulation of cell death. Heatmap and gene expression trend linear model of representative GO Term "Negative Regulation Of Neuron Death (GO:1901215)". B) Bubble plot highlighting unique GO Terms to Cluster 2, relating to immune response. Heatmap and gene expression trend linear model of representative GO Term "Positive Regulation Of Immune Response (GO:0050778)". C) Bubble plot highlighting unique GO Terms to Cluster 3, relating to inflammation response and metabolism. Heatmap and gene expression trend linear model of representative GO Term "Positive Regulation Of Cellular Metabolic Process (GO:0031325)". D) Bubble plot highlighting unique GO Terms to Cluster 4, relating to neurotrophic signaling and extracellular matrix. Heatmap and gene expression trend linear model of representative GO Term "Positive Regulation Of Cell-Cell Adhesion Mediated By Cadherin (GO:2000049)".

genes regulating these pathways in T21 foreshadow neurodevelopmental defects in DS and suggest that there may be increased neuronal death throughout neural development in DS. Cluster 2 was enriched for terms related to innate immune response (Fig. 4B). Interactions between the immune system and neuronal cells influence processes critical for neural development, including synapse remodeling. The onset and impact of decreased gene expression in these pathways on neurodevelopment in T21 remain unclear as our culture paradigm does not include interactions with immune cells. Nonetheless, the results suggest that T21 neural progenitor cells have intrinsic dysregulation that may impact response to inflammation early in neural induction. Cluster 3 shows enrichment of genes in inflammation response and metabolism pathways (Fig. 4C), indicating T21 causes an early dysregulation of metabolic processes. Cluster 4 highlights a dysregulation in the extracellular matrix (ECM) early in neural induction (Fig. 4D). Disruptions in ECM can affect many aspects of neural development (Long and Huttner, 2019), and these results may predict multiple processes that are defective in DS and other neural development disorders (Long and Huttner, 2021).

The impact of T21 on WNT signaling emerges from analysis of clusters 1, 2 and 3, so we specifically explored the biological impact of WNT signaling pathways in our data. Using a lower threshold of an adjusted p-value<0.05 revealed dysregulation in both canonical and non-canonical WNT signaling pathways in T21 (Fig. 5). Four genes were differentially expressed in the non-canonical WNT signaling pathways (Fig. 5A), while three of four DEGs in the non-canonical pathway overlapped with the canonical pathway genes (Fig. 5). The trajectories of these four genes indicated an increase in expression in T21 compared to euploid during the course of neural induction (Fig 5A). Analysis of canonical (β-catenin) WNT signaling identified 18 genes as differentially expressed in T21 at all timepoints (Fig. 5B), with all 18 genes showing a trend toward

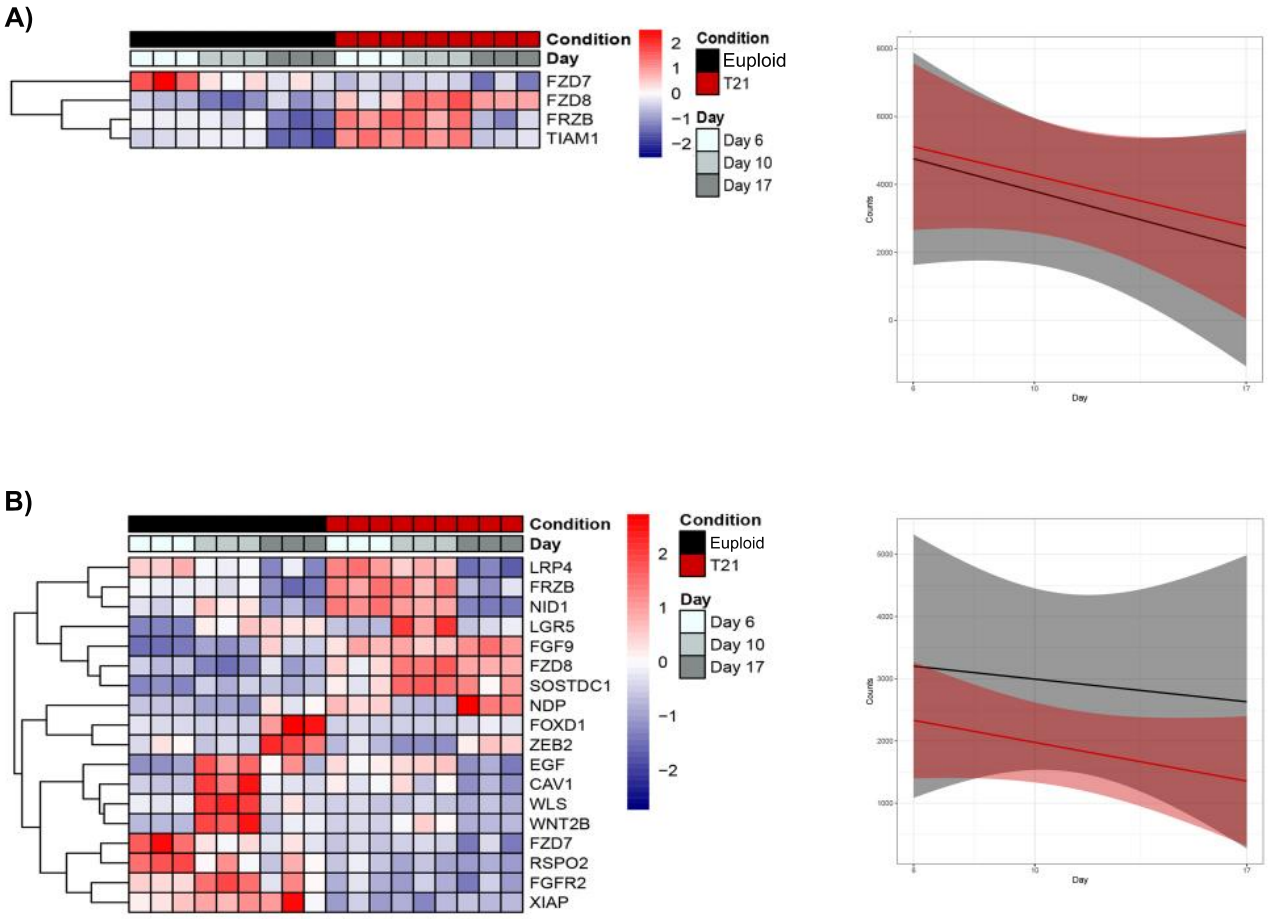

Figure 5. Reciprocal gene expression in Canonical versus Non-Canonical WNT Signaling. A) Heatmap and plot showing linear model of the average expression trends of differentially expressed Canonical WNT signaling pathway genes that have an adjusted p-value<0.05. Time course replicates are labeled with light gray for day 6, medium gray day 10, and dark gray day 17. Isogenic control is indicated by black and T21 by red. Upregulated genes are displayed in red while downregulated genes are expressed in blue. B) Heatmap and plot showing linear model of the average expression trends of differentially expressed Non-Canonical WNT signaling pathway genes that have an adjusted p-value<0.05. Time course replicates are labeled with light gray for day 6, medium gray day 10, and dark gray day 17. Isogenic Control is highlighted by black and T21 by red. Upregulated genes are displayed in red while downregulated genes are expressed in blue.

decreased expression throughout differentiation in both T21 and euploid, but an overall decrease in expression in T21 compared to euploid. These results suggest that non-canonical and canonical WNT signaling are reciprocally affected by T21.

Discussion

Transcriptional dysregulation by T21 during neural induction

We leveraged *in vitro* iPSC models of Down syndrome to assess the transcriptional impact of T21 on the process of neural induction, building on seminal work modeling *in vivo* human neural development with hESCs (Lavaute et al., 2009, Pankratz et al., 2007, Zhang et al., 2001). These stages are inaccessible in human embryos, although the emergence of PSC-derived embryo models could eventually provide an opportunity to assess how the presence of an extra chromosome affects early embryology and, potentially, neural induction (Weatherbee et al., 2023, Pedroza et al., 2023, Oldak et al., 2023). To our knowledge this is the first study to explore the earliest stages of neural induction in T21.

We used isogenic T21 and euploid control iPSCs to avoid natural genetic variation between individuals that generates transcriptomic variation, enabling more sensitive assignment of causal relationships between genotype and gene expression (Weick et al., 2013a, Murray et al., 2015b). Of the 300 proteins encoded on HSA21, many are dosage sensitive which means their gene dosage may be driving phenotypes in DS. However, we do not have a complete picture of which of these 300 protein-coding genes are or are not dose sensitive, making it difficult to attribute Down syndrome phenotypes to any one gene or small groups of genes (e.g. drivers). In our analysis, we were somewhat surprised to find that only a small number of HSA21 genes have altered expression. Other transcriptomic studies focused on Down syndrome and neural/brain development observe larger proportions of HSA21 genes as DEGs (Olmos-Serrano et al., 2016,

Sobol et al., 2019, Meharena et al., 2022, Li et al., 2022). Our results may be due to our stringent parameters or differences in stage of development. The low percentage of affected HSA21 genes and early global transcriptomic changes during neural induction suggest mechanisms beyond HSA21 specific genes as drivers of early neurodevelopment defects.

We found only three HSA21 genes – *CHODL*, *PCP4*, and *TTC3* –were consistently altered throughout the process of neural induction. All three have roles in neural development and function, implicated in processes such as neurite outgrowth, that are related to neuron differentiation and maturation (Sleigh et al., 2014, Zhong et al., 2012, Kitazono et al., 2020, Mouton-Liger et al., 2011, Mouton-Liger et al., 2014, Endo et al., 2023, Berto et al., 2007, Berto et al., 2014). The increased expression of these genes in T21 cells as they differentiate may suggest that T21 cells are undergoing heterochronic, or early, differentiation as has been suggested by other transcriptomic studies (Zhou et al., 2023, Li et al., 2022).

TTC3 has also been implicated in pathways crucial for proper maintenance of the proteasome, including the ribosomal quality control (RQC) system (Endo et al., 2023), and the ubiquitin-proteasome system (Zhou et al., 2022), which also appeared in our functional analysis in Cluster 3 (Fig. 4C) Overexpression of *TTC3* can aggregate TTC3 protein, resulting in loss of its physiologic ubiquitin-ligase activity. This aggregation may simultaneously promote aggregation of pathological proteins, such as amyloid beta, while also causing inappropriate segregation of proteins crucial for mitochondrial function, such as DNA polymerase γ (POLG) (Zhou et al., 2022). During periods of translational stress when TTC3 is physiologically upregulated, it interacts with the 40S ribosomal subunit to repress translation of transcripts associated with metabolic processes required for cellular homeostasis (Endo et al., 2023). Taken together, this early upregulation of TTC3 may contribute to known metabolic dysfunction through promotion of

mitochondrial stress and downregulation of key homeostatic processes and later protein aggregation associated with Alzheimer's disease development in individuals with T21 (Lanzillotta and Di Domenico, 2021). However, future work is needed to determine if TTC3 is a direct mechanism of metabolic stress in T21 and to determine if TTC3 upregulation is an effect of gene dosage or is an early indication of broader trisomy associated translational stress.

Emergence of dysregulated pathways previously implicated in Down syndrome during neural induction.

Reduced cortical size, hypocellularity and defects in neurogenesis have been established in mid-late gestation fetuses and neonates with T21 (Wisniewski et al., 1984, Schmidt-Sidor et al., 1990, Guidi et al., 2008, Guidi et al., 2011, Guidi et al., 2018, Stagni et al., 2019, Patkee et al., 2020, Baburamani et al., 2020, Tarui et al., 2020, Golden and Hyman, 1994, Ross et al., 1984, McCann et al., 2021, Guihard-Costa et al., 2006). Several non-exclusive hypotheses have emerged to explain these deficits including that T21 results in fewer progenitors and/or reduced progenitor proliferation, decreased migration of progenitors, or increased progenitor death (Giffin-Rao et al., 2022, Huo et al., 2018a, Lu et al., 2012). Our results shed some mechanistic light on the impact of T21 on the establishment of the nervous system, and demonstrate that T21 dysregulates genes involved in pathways relevant to the phenotypes of individuals with DS and cells from these individuals including oxidative stress, morphological abnormalities, immune disorders, divergent metabolic states, and premature aging (Muchová et al., 2014, Coskun and Busciglio, 2012).

Divergent metabolic states impact neurogenesis (Traxler et al., 2021, Zheng et al., 2016, Iwata and Vanderhaeghen, 2021). We observe an increased regulation of Positive Cellular Metabolic Process and Positive Regulation of Reactive Oxygen Species Metabolic Process as

potential drivers for deficits in neural development in T21. These findings are supported by previous analyses of T21 neural progenitor cells having increase metabolic requirements due to mitochondrial stress (Prutton et al., 2023) and increased oxidative stress (Jovanovic et al., 1998). Importantly, our data indicate that T21 driven metabolic dysfunction emerges early in neural development and thus may impact altered neurogenesis in DS.

Our results suggest that ECM pathways are aberrant in DS. During neural development, neural stem cells and progenitors must precisely regulate their morphology to allow for proper function. The ECM is a key regulator in this process, modulating cell adhesion, shape, proliferation, differentiation, migration and morphogenesis of neural tissues (Long and Huttner, 2019). The HSA21 gene RUNX1 has been implicated in the regulation of the extracellular matrix in DS leading to a decrease in cell migration (Mollo et al., 2022). T21-driven increased cell-cell adhesion early in neural induction may lead to reduced migration and proliferation, ultimately affecting the process of neural development.

Overall, our results unveil early dysregulation of pathways that have been previously implicated in neural development and function in DS. Increases in oxidative stress and interferons, as well as cell death, likely impact the generation of progenitors and neuron differentiation.

WNT genes are dysregulated by T21

WNTs are a highly conserved group of secreted factors that function in a tightly regulated temporal- and spatial-specific manner during forebrain development to regulate proliferation, differentiation, and regional identity (Tiberi et al., 2012, Mulligan and Cheyette, 2012, Clevers, 2006). WNT signaling controls initial formation of the neural plate and subsequent patterning decisions in the embryonic nervous system (Patapoutian and Reichardt, 2000, Leung et al.,

2016). WNTs signal through β-catenin in the canonical pathway, or through non-canonical pathways that mediate calcium (Liu et al., 2022). The distinct contribution of these two modes of WNT signaling on stem cell maintenance and neural differentiation is confounded by the promiscuity of ligands and receptors across pathways and cross talk with other signaling pathways (Bengoa-Vergniory and Kypta, 2015). Our transcriptional data indicate that WNT signaling gene expression is dysregulated in T21 during neural induction and that both canonical and non-canonical WNT signaling pathway genes are affected by T21 (Fig. 5).

T21 cells have alterations in genes in the non-canonical WNT pathway. Non-canonical WNT signaling is critical in early neural induction (Huang et al., 2016, Lee et al., 2015, Bengoa-Vergniory et al., 2014) and promotion of asymmetrical progenitor division (Delaunay et al., 2014, Lake and Sokol, 2009). Furthermore, suppression of the non-canonical WNT pathway is necessary for generation of later born, upper layer neurons (Delaunay et al., 2014, Lake and Sokol, 2009), potentially implicating early elevation in the activity of the non-canonical WNT pathway in the reduced number of upper layer neurons in DS (Golden and Hyman, 1994, Giffin-Rao et al., 2022). Interestingly, the only unique gene identified in this pathway was TIAM1, an HSA21 encoded gene. TIAM1 is a mediator of Disheveled and Rac1 interaction required for Wnt5a-induced signaling transduction (Čajánek et al., 2013). Knockdown of TIAM1 causes impaired neuron generation (Čajánek et al., 2013), suggesting that upregulation in T21 may force early neural differentiation compared to euploid cells. The specific contribution of TIAM1 upregulation in T21 on neural differentiation and cognitive phenotypes will need to be further explored.

Our data are consistent with the existing body of literature showing a decrease in canonical WNT pathway signaling over the course of neural induction (Huang et al., 2016,

Heeg-Truesdell and LaBonne, 2006, Telias and Ben-Yosef, 2021). Of note, we did not externally modulate WNT signaling with XAV939, an antagonist of the canonical pathway known to promote neural specification. There is a clear downregulation of canonical WNT pathway gene expression across all stages of neural induction in T21 derived cells compared to euploid (Fig. 5A).

Canonical WNT signaling has also been associated with promotion of stem cell proliferation and inhibition of progenitor apoptosis (Dravid et al., 2005, Davidson et al., 2007, Lee et al., 2015). The decrease in canonical WNT signaling in our results correlates with decreased cell proliferation in T21 (Sobol et al., 2019, Sharma et al., 2022). Overall, given the critical role of the canonical WNT signaling pathway in maintaining stemness and proliferation, these data may indicate that T21 cells are less responsive to cues to maintain or expand progenitor pools in the brain. It will be important to test whether WNT activation alters early neural progenitor subpopulations through further study of T21 cell cycle and differentiation dynamics following selective canonical agonism with small molecule inhibitors like CHIR99021 or over expression of non-degradable β-catenin.

These data are consistent with previous work by our lab and others showing a downregulation of canonical WNT signaling in T21 (Granno et al., 2019, Giffin-Rao et al., 2022, Qiu et al., 2023, Hibaoui et al., 2014). DYRK1A, a HSA21 dosage sensitive gene, interacts with the canonical pathway through GSK3β to inhibit pathway activity (Qiu et al., 2023, Hibaoui et al., 2014). However, our data does not show consistent upregulation of DYRK1A in trisomic cells during neural induction suggesting that T21 may have a broader effect on the canonical WNT pathway beyond DYRK1A.

Conclusion

This comprehensive analysis reveals that trisomy 21 impacts discrete developmental pathways at the earliest stages of neural development. Further, the results suggest that metabolic dysfunction arises early in embryogenesis in trisomy 21 and may thus affect development and function more broadly.

Author Contributions

JLM: Writing – drafting, reviewing, & editing, Acquisition of data, Analysis of data, Interpretation of data, Conceptualization, Methodology; JGP: Writing – drafting, reviewing, & editing, Analysis of data, Interpretation of data; MC: Writing – drafting, & editing, Acquisition of data, Analysis of data, Methodology; IAS: Acquisition of data, Analysis of data; NM: Analysis of data; CLS: Writing – reviewing, & editing, Methodology – RNAseq analysis; YG-R: Acquisition of data, Conceptualization, Methodology; AB: Writing – drafting, reviewing, & editing, Interpretation of data, Conceptualization, Methodology, Project Administration

Funding

The work in this project was supported by the funding from Eunice Kennedy Shriver National Institute of Child Health and Human Development (1R01HD106197-01 and 1R01HD111938-01, to AB), the University of Wisconsin-Madison School of Medicine and Public Health (AB), Wisconsin Alumni Research Foundation (AB), and by a core grant to the Waisman Center from the Eunice Kennedy Shriver National Institute of Child Health and Human Development (P50HD105353). JLM was supported by an R36 Dissertator Grant from the National Institute of Aging (R36AG072108), the Advanced Opportunity Fellowship - SciMed Graduate Research Scholars at the University of Wisconsin-Madison, the University of Wisconsin Madison Biotechnology Training Program (NIH 5 T32 GM135066), and a fellowship

from The Morse Society at the Waisman Center. JGP was supported by a grant to the University of Wisconsin Madison Medical Scientist Training Program (T32 GM140935).

Acknowledgments

We thank members of laboratories of Su-Chun Zhang and André Sousa for valuable feedback. We also thank Robin Dowell D.Sc., Mary Allen Ph.D., and Joaquin M Espinosa Ph.D. and The Include Data Coordinating Center's Data Science for Diverse Scholars in Down Syndrome Research (DS3) program, for valuable computational analysis training.

Data Availability Statement

The datasets generated and analyzed for this study can be found in the Gene Expression Omnibus (GEO) database, GSE247990.

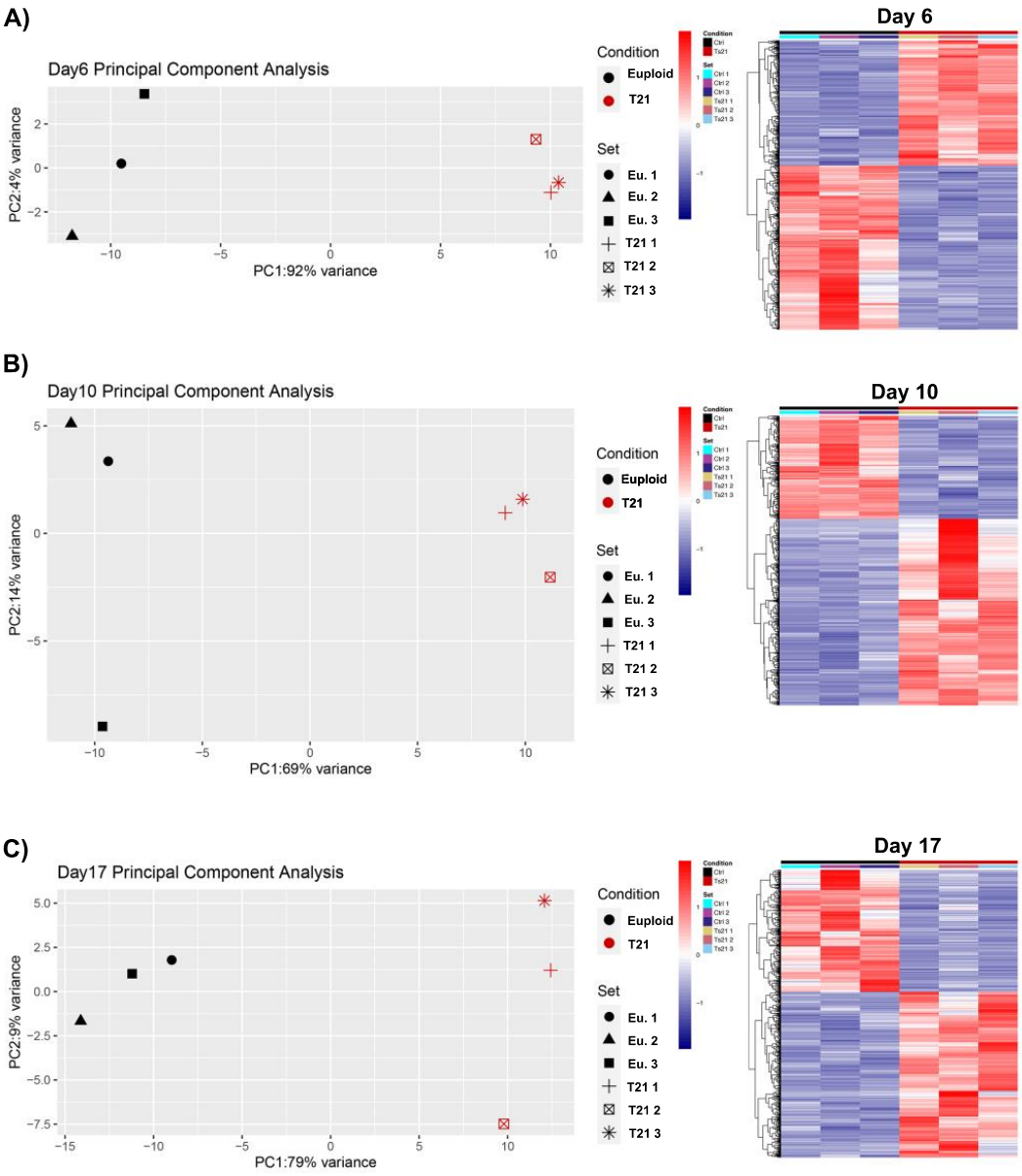

Figure S1. Gene expression differences between isogenic Trisomy 21 and euploid cells at each time-point. Principal Component Analysis of Trisomy21 (red) and isogenic control (black), showing PC1 variance as condition at A) Day 6, B) day 10 and C) day 17. Heatmaps of expression profiles showing clear clustering of T21 versus isogenic control at each timepoint. Time course replicates are labeled with light gray for day 6, medium gray day 10, and dark gray day 17. Isogenic Control is highlighted by black and T21 by red. Upregulated genes are displayed in red while downregulated genes are expressed in blue.

REFERENCES

2021. 2021 Alzheimer's disease facts and figures. *Alzheimer's & Dementia,* 17**,** 327-406.

AKESON, E. C., LAMBERT, J. P., NARAYANSWAMI, S., GARDINER, K., BECHTEL, L. J. & DAVISSON, M. T. 2001. Ts65Dn -- localization of the translocation breakpoint and trisomic gene content in a mouse model for Down syndrome. *Cytogenet.Cell Genet.,* 93**,** 270-276.

ALLAWAY, K. C. & MACHOLD, R. 2017. Developmental specification of forebrain cholinergic neurons. *Developmental Biology,* 421**,** 1-7.

ALLDRED, M. J., CHAO, H. M., LEE, S. H., BEILIN, J., POWERS, B. E., PETKOVA, E., STRUPP, B. J. & GINSBERG, S. D. 2018. CA1 pyramidal neuron gene expression mosaics in the Ts65Dn murine model of Down syndrome and Alzheimer's disease following maternal choline supplementation. *Hippocampus,* 28**,** 251-268.

ALLDRED, M. J., CHAO, H. M., LEE, S. H., BEILIN, J., POWERS, B. E., PETKOVA, E., STRUPP, B. J. & GINSBERG, S. D. 2019. Long-term effects of maternal choline supplementation on CA1 pyramidal neuron gene expression in the Ts65Dn mouse model of Down syndrome and Alzheimer's disease. *Faseb Journal,* 33**,** 9871-9884.

ALLDRED, M. J., PENIKALAPATI, S. C., LEE, S. H., HEGUY, A., ROUSSOS, P. & GINSBERG, S. D. 2021. Profiling Basal Forebrain Cholinergic Neurons Reveals a Molecular Basis for Vulnerability Within the Ts65Dn Model of Down Syndrome and Alzheimer's Disease. *Mol Neurobiol,* 58**,** 5141-5162.

ALLDRED, M. J., PIDIKITI, H., HEGUY, A., ROUSSOS, P. & GINSBERG, S. D. 2023. Basal forebrain cholinergic neurons are vulnerable in a mouse model of Down syndrome and their molecular fingerprint is rescued by maternal choline supplementation. *The FASEB Journal,* 37**,** e22944.

ALONSO, A. D., COHEN, L. S., CORBO, C., MOROZOVA, V., ELIDRISSI, A., PHILLIPS, G. & KLEIMAN, F. E. 2018. Hyperphosphorylation of Tau Associates With Changes in Its Function Beyond Microtubule Stability. *Front Cell Neurosci,* 12**,** 338.

ALONSO, A. D., DI CLERICO, J., LI, B., CORBO, C. P., ALANIZ, M. E., GRUNDKE-IQBAL, I. & IQBAL, K. 2010. Phosphorylation of tau at Thr212, Thr231, and Ser262 combined causes neurodegeneration. *Journal of Biological Chemistry,* 285**,** 30851-30860.

AMANO, T., JEFFRIES, E., AMANO, M., KO, A. C., YU, H. & KO, M. S. 2015. Correction of Down syndrome and Edwards syndrome aneuploidies in human cell cultures. *DNA Res,* 22**,** 331-42.

ANDREADIS, A., BROWN, W. M. & KOSIK, K. S. 1992. STRUCTURE AND NOVEL EXONS OF THE HUMAN-TAU GENE. *Biochemistry,* 31**,** 10626-10633.

ANTONARAKIS, S. E. 2001. Chromosome 21: from sequence to applications. *Curr.Opin.Genet Dev.,* 11**,** 241-246.

AQUILONIUS, S. M., ECKERNÅS, S. A. & SUNDWALL, A. 1975. Regional distribution of choline acetyltransferase in the human brain: changes in Huntington's chorea. *J Neurol Neurosurg Psychiatry,* 38**,** 669-77.

ARENDT, T., BIGL, V., ARENDT, A. & TENNSTEDT, A. 1983. Loss of neurons in the nucleus basalis of Meynert in Alzheimer's disease, paralysis agitans and Korsakoff's Disease. *Acta Neuropathol,* 61**,** 101-8.

ARENDT, T., BIGL, V., TENNSTEDT, A. & ARENDT, A. 1985. Neuronal loss in different parts of the nucleus basalis is related to neuritic plaque formation in cortical target areas in Alzheimer's disease. *Neuroscience,* 14**,** 1-14.

AREOSA, S. A. & SHERRIFF, F. 2003. Memantine for dementia. *The Cochrane database of systematic reviews***,** CD003154-CD003154.

ASBREUK, C. H., VAN SCHAICK, H. S., COX, J. J., KROMKAMP, M., SMIDT, M. P. & BURBACH, J. P. 2002. The homeobox genes Lhx7 and Gbx1 are expressed in the basal forebrain cholinergic system. *Neuroscience,* 109**,** 287-98.

ASH, J. A., VELAZQUEZ, R., KELLEY, C. M., POWERS, B. E., GINSBERG, S. D., MUFSON, E. J. & STRUPP, B. J. 2014. Maternal choline supplementation improves spatial mapping and increases basal forebrain cholinergic neuron number and size in aged Ts65Dn mice. *Neurobiol Dis,* 70**,** 32-42.

ASSOCIATION, A. S. 2019. FDA-approved treatments for Alzheimer’s.

AULD, D. S., MENNICKEN, F. & QUIRION, R. 2001. Nerve growth factor rapidly induces prolonged acetylcholine release from cultured basal forebrain neurons: differentiation between neuromodulatory and neurotrophic influences. *J Neurosci,* 21**,** 3375-82.

BABURAMANI, A. A., VONTELL, R. T., UUS, A., PIETSCH, M., PATKEE, P. A., WYATT-ASHMEAD, J., CHIN-SMITH, E. C., SUPRAMANIAM, V. G., DONALD TOURNIER, J., DEPREZ, M. & RUTHERFORD, M. A. 2020. Assessment of radial glia in the frontal lobe of fetuses with Down syndrome. *Acta neuropathologica communications,* 8**,** 141-141.

BAKER, D. J. & PETERSEN, R. C. 2018. Cellular senescence in brain aging and neurodegenerative diseases: evidence and perspectives. *Journal of Clinical Investigation,* 128**,** 1208-1216.

BAKER-NIGH, A., VAHEDI, S., DAVIS, E. G., WEINTRAUB, S., BIGIO, E. H., KLEIN, W. L. & GEULA, C. 2015a. Neuronal amyloid-beta accumulation within cholinergic basal forebrain in ageing and Alzheimer's disease. 138**,** 1737.

BAKER-NIGH, A., VAHEDI, S., DAVIS, E. G., WEINTRAUB, S., BIGIO, E. H., KLEIN, W. L. & GEULA, C. 2015b. Neuronal amyloid-β accumulation within cholinergic basal forebrain in ageing and Alzheimer’s disease. *Brain,* 138**,** 1722-1737.

BALLINGER, E. C., ANANTH, M., TALMAGE, D. A. & ROLE, L. W. 2016. Basal Forebrain Cholinergic Circuits and Signaling in Cognition and Cognitive Decline. *Neuron,* 91**,** 1199-1218.

BARBACID, M. 1994. The Trk family of neurotrophin receptors. *J Neurobiol,* 25**,** 1386-403.

BARRON, S. A., MAZLIAH, J. & BENTAL, E. 1987. Sympathetic cholinergic dysfunction in amyotrophic lateral sclerosis. *Acta Neurol Scand,* 75**,** 62-3.

BATEMAN, R. J., XIONG, C., BENZINGER, T. L., FAGAN, A. M., GOATE, A., FOX, N. C., MARCUS, D. S., CAIRNS, N. J., XIE, X. & BLAZEY, T. M. 2012. Clinical and biomarker changes in dominantly inherited Alzheimer's disease. *N Engl J Med,* 367**,** 795-804.

BENGOA-VERGNIORY, N., GORROÑO-ETXEBARRIA, I., GONZÁLEZ-SALAZAR, I. & KYPTA, R. M. 2014. A switch from canonical to noncanonical Wnt signaling mediates early differentiation of human neural stem cells. *Stem Cells,* 32**,** 3196-208.

BENGOA-VERGNIORY, N. & KYPTA, R. M. 2015. Canonical and noncanonical Wnt signaling in neural stem/progenitor cells. *Cell Mol Life Sci,* 72**,** 4157-72.

BENTHAM, P., GRAY, R., RAFTERY, J., HILLS, R., SELLWOOD, E., COURTNEY, C., FARRELL, D., HARDYMAN, W., CROME, P., EDWARDS, S., LENDON, C., LYNCH, L. & GRP, A. D. C. 2004. Long-term donepezil treatment in 565 patients with Alzheimer's disease (AD2000): randomised double-blind trial. *Lancet,* 363**,** 2105-2115.

BERTO, G., CAMERA, P., FUSCO, C., IMARISIO, S., AMBROGIO, C., CHIARLE, R., SILENGO, L. & DI CUNTO, F. 2007. The Down syndrome critical region protein TTC3 inhibits neuronal differentiation via RhoA and Citron kinase. *J Cell Sci,* 120**,** 1859-67.

BERTO, G. E., IOBBI, C., CAMERA, P., SCARPA, E., IAMPIETRO, C., BIANCHI, F., GAI, M., SGRÒ, F., CRISTOFANI, F., GÄRTNER, A., DOTTI, C. G. & DI CUNTO, F. 2014. The DCR protein TTC3 affects differentiation and Golgi compactness in neurons through specific actin-regulating pathways. *PLoS One,* 9**,** e93721.

BEYREUTHER, K. & MASTERS, C. L. 1995. NEURODEGENERATION AND DEMENTIA - ALZHEIMERS-DISEASE AS A MODEL. *Arzneimittel-Forschung/Drug Research,* 45-1**,** 347-350.

BIERER, L. M., HAROUTUNIAN, V., GABRIEL, S., KNOTT, P. J., CARLIN, L. S., PUROHIT, D. P., PERL, D. P., SCHMEIDLER, J., KANOF, P. & DAVIS, K. L. 1995. Neurochemical correlates of dementia severity in Alzheimer's disease: relative importance of the cholinergic deficits. *J Neurochem,* 64**,** 749-60.

BISSONNETTE, C. J., LYASS, L., BHATTACHARYYA, B. J., BELMADANI, A., MILLER, R. J. & KESSLER, J. A. 2011. The controlled generation of functional basal forebrain cholinergic neurons from human embryonic stem cells. *Stem Cells,* 29**,** 802-11.

BITTLES, A. H. & GLASSON, E. J. 2004. Clinical, social, and ethical implications of changing life expectancy in Down syndrome. *Dev Med Child Neurol,* 46**,** 282-6.

BLANCO-CENTURION, C., GERASHCHENKO, D. & SHIROMANI, P. J. 2007. Effects of saporin-induced lesions of three arousal populations on daily levels of sleep and wake. *J Neurosci,* 27**,** 14041-8.

BOSKOVIC, Z., MEIER, S., WANG, Y., MILNE, MICHAEL R., ONRAET, T., TEDOLDI, A. & COULSON, ELIZABETH J. 2019. Regulation of cholinergic basal forebrain development, connectivity, and function by neurotrophin receptors. *Neuronal Signaling,* 3.

BOTHWELL, M. 2014. NGF, BDNF, NT3, and NT4. *Handb Exp Pharmacol,* 220**,** 3-15.

BOTLY, L. C. & DE ROSA, E. 2009. Cholinergic deafferentation of the neocortex using 192 IgG-saporin impairs feature binding in rats. *Journal of Neuroscience,* 29**,** 4120-4130.

BOTTÉ, A., LAINÉ, J., XICOTA, L., HEILIGENSTEIN, X., FONTAINE, G., KASRI, A., RIVALS, I., GOH, P., FAKLARIS, O., COSSEC, J. C., MOREL, E., REBILLAT, A. S., NIZETIC, D., RAPOSO, G. & POTIER, M. C. 2020. Ultrastructural and dynamic studies of the endosomal compartment in Down syndrome. *Acta Neuropathol Commun,* 8**,** 89.

BRAAK, E., GRIFFING, K., ARAI, K., BOHL, J., BRATZKE, H. & BRAAK, H. 1999. Neuropathology of Alzheimer's disease: what is new since A. Alzheimer? *Eur Arch Psychiatry Clin Neurosci,* 249 Suppl 3**,** 14-22.

BRAAK, H. & BRAAK, E. 1991. Neuropathological stageing of Alzheimer-related changes. *Acta neuropathologica,* 82**,** 239-259.

BRAAK, H. & BRAAK, E. 1996. Evolution of the neuropathology of Alzheimer's disease. *Acta Neurol Scand Suppl,* 165**,** 3-12.

BRAAK, H. & DEL TREDICI, K. 2011. The pathological process underlying Alzheimer's disease in individuals under thirty. *Acta neuropathologica,* 121**,** 171-181.

BRAZEL, C. Y., ROMANKO, M. J., ROTHSTEIN, R. P. & LEVISON, S. W. 2003. Roles of the mammalian subventricular zone in brain development. *Progress in Neurobiology,* 69**,** 49-69.

BRIGGS, J. A., SUN, J., SHEPHERD, J., OVCHINNIKOV, D. A., CHUNG, T. L., NAYLER, S. P., KAO, L. P., MORROW, C. A., THAKAR, N. Y., SOO, S. Y., PEURA, T., GRIMMOND, S. & WOLVETANG, E. J. 2013. Integration-free induced pluripotent stem cells model genetic and neural developmental features of down syndrome etiology. *Stem Cells,* 31**,** 467-78.

BUDNI, J., BELLETTINI-SANTOS, T., MINA, F., GARCEZ, M. L. & ZUGNO, A. I. 2015. The involvement of BDNF, NGF and GDNF in aging and Alzheimer's disease. *Aging Dis,* 6**,** 331-41.

BURT, D. B., LOVELAND, K. A., PRIMEAUX-HART, S., CHEN, Y. W., PHILLIPS, N. B., CLEVELAND, L. A., LEWIS, K. R., LESSER, J. & CUMMINGS, E. 1998. Dementia in adults with Down syndrome: Diagnostic challenges. *American Journal on Mental Retardation,* 103**,** 130-145.

BUSCIGLIO, J. & YANKNER, B. A. 1995. Apoptosis and increased generation of reactive oxygen species in Down's syndrome neurons in vitro. *Nature,* 378**,** 776-779.

BUTCHER, L. L. & SEMBA, K. 1989. REASSESSING THE CHOLINERGIC BASAL FOREBRAIN - NOMENCLATURE SCHEMATA AND CONCEPTS. *Trends in Neurosciences,* 12**,** 483-485.

CACABELOS, R. 2007. Donepezil in Alzheimer's disease: From conventional trials to pharmacogenetics. *Neuropsychiatric Disease and Treatment.*

ČAJÁNEK, L., GANJI, R. S., HENRIQUES-OLIVEIRA, C., THEOFILOPOULOS, S., KONÍK, P., BRYJA, V. & ARENAS, E. 2013. Tiam1 regulates the Wnt/Dvl/Rac1 signaling pathway and the differentiation of midbrain dopaminergic neurons. *Mol Cell Biol,* 33**,** 59-70.

CALSOLARO, V. & EDISON, P. 2016. Neuroinflammation in Alzheimer's disease: current evidence and future directions. *Alzheimer's & dementia,* 12**,** 719-732.

CASANOVA, E. L. & CASANOVA, M. F. 2014. Genetics studies indicate that neural induction and early neuronal maturation are disturbed in autism. *Front Cell Neurosci,* 8**,** 397.

CASANOVA, M. F., WALKER, L. C., WHITEHOUSE, P. J. & PRICE, D. L. 1985. Abnormalities of the nucleus basalis in Down's syndrome. *Ann Neurol,* 18**,** 310-3.

CHAMBERS, S. M., FASANO, C. A., PAPAPETROU, E. P., TOMISHIMA, M., SADELAIN, M. & STUDER, L. 2009. Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. *Nat Biotechnol,* 27**,** 275-80.

CHANG, C. Y., CHEN, S. M., LU, H. E., LAI, S. M., LAI, P. S., SHEN, P. W., CHEN, P. Y., SHEN, C. I., HARN, H. J., LIN, S. Z., HWANG, S. M. & SU, H. L. 2015. N-butylidenephthalide Attenuates Alzheimer's Disease-Like Cytopathy in Down Syndrome Induced Pluripotent Stem Cell-Derived Neurons. *Scientific Reports,* 5.

CHEN, C., JIANG, P., XUE, H., PETERSON, S. E., TRAN, H. T., MCCANN, A. E., PARAST, M. M., LI, S., PLEASURE, D. E. & LAURENT, L. C. 2014. Role of astroglia in Down's syndrome revealed by patient-derived human-induced pluripotent stem cells. *Nature communications,* 5**,** 1-18.

CHEN, E. Y., TAN, C. M., KOU, Y., DUAN, Q., WANG, Z., MEIRELLES, G. V., CLARK, N. R. & MA'AYAN, A. 2013. Enrichr: interactive and collaborative HTML5 gene list enrichment analysis tool. *BMC Bioinformatics,* 14**,** 128.

CHEN, X.-Q. & MOBLEY, W. C. 2019. Exploring the Pathogenesis of Alzheimer Disease in Basal Forebrain Cholinergic Neurons: Converging Insights From Alternative Hypotheses. *Frontiers in Neuroscience,* 13.

CHEN, X. Q., SAWA, M. & MOBLEY, W. C. 2018. Dysregulation of neurotrophin signaling in the pathogenesis of Alzheimer disease and of Alzheimer disease in Down syndrome. *Free Radical Biology and Medicine,* 114**,** 52-61.

CHEN, Y., CAO, J., XIONG, M., PETERSEN, A. J., DONG, Y., TAO, Y., HUANG, C. T., DU, Z. & ZHANG, S. C. 2015. Engineering Human Stem Cell Lines with Inducible Gene Knockout using CRISPR/Cas9. *Cell Stem Cell,* 17**,** 233-44.

CHENG, L., XU, C., WANG, L., AN, D., JIANG, L., ZHENG, Y., XU, Y., WANG, Y., WANG, Y., ZHANG, K., WANG, X., ZHANG, X., BAO, A., ZHOU, Y., YANG, J., DUAN, S., SWAAB, D. F., HU, W. & CHEN, Z. 2021. Histamine H(1) receptor deletion in cholinergic neurons induces sensorimotor gating ability deficit and social impairments in mice. *Nat Commun,* 12**,** 1142.

CHIEFFI, S., CAROTENUTO, M., MONDA, V., VALENZANO, A., VILLANO, I., PRECENZANO, F., TAFURI, D., SALERNO, M., FILIPPI, N., NUCCIO, F., RUBERTO, M., DE LUCA, V., CIPOLLONI, L., CIBELLI, G., MOLLICA, M. P., IACONO, D., NIGRO, E., MONDA, M., MESSINA, G. & MESSINA, A. 2017. Orexin System: The Key for a Healthy Life. *Frontiers in Physiology,* 8.

CHO, H. H., CARGNIN, F., KIM, Y., LEE, B., KWON, R. J., NAM, H., SHEN, R., BARNES, A. P., LEE, J. W., LEE, S. & LEE, S. K. 2014. Isl1 directly controls a cholinergic neuronal identity in the developing forebrain and spinal cord by forming cell type-specific complexes. *PLoS Genet,* 10**,** e1004280.

CHOU, S. T., BYRSKA-BISHOP, M., TOBER, J. M., YAO, Y., VANDORN, D., OPALINSKA, J. B., MILLS, J. A., CHOI, J. K., SPECK, N. A., GADUE, P., HARDISON, R. C., NEMIROFF, R. L., FRENCH, D. L. & WEISS, M. J. 2012. Trisomy 21-associated defects in human primitive hematopoiesis revealed through induced pluripotent stem cells. *Proceedings of the National Academy of Sciences of the United States of America,* 109**,** 17573-17578.

CLARKE, Z. A., ANDREWS, T. S., ATIF, J., POUYABAHAR, D., INNES, B. T., MACPARLAND, S. A. & BADER, G. D. 2021. Tutorial: guidelines for annotating single-cell transcriptomic maps using automated and manual methods. *Nature Protocols,* 16**,** 2749-2764.

CLAVAGUERA, F., BOLMONT, T., CROWTHER, R. A., ABRAMOWSKI, D., FRANK, S., PROBST, A., FRASER, G., STALDER, A. K., BEIBEL, M., STAUFENBIEL, M., JUCKER, M., GOEDERT, M. & TOLNAY, M. 2009. Transmission and spreading of tauopathy in transgenic mouse brain. *Nature Cell Biology,* 11**,** 909-U325.

CLAYTON, K. A., VAN ENOO, A. A. & IKEZU, T. 2017. Alzheimer's disease: the role of microglia in brain homeostasis and proteopathy. *Frontiers in neuroscience,* 11**,** 680.

CLEVERS, H. 2006. Wnt/β-Catenin Signaling in Development and Disease. *Cell,* 127**,** 469-480.

COLACURCIO, D. J., PENSALFINI, A., JIANG, Y. & NIXON, R. A. 2018. Dysfunction of autophagy and endosomal-lysosomal pathways: Roles in pathogenesis of Down syndrome and Alzheimer's Disease. *Free Radic Biol Med,* 114**,** 40-51.

CONNER, J. M., CULBERSON, A., PACKOWSKI, C., CHIBA, A. A. & TUSZYNSKI, M. H. 2003. Lesions of the Basal forebrain cholinergic system impair task acquisition and abolish cortical plasticity associated with motor skill learning. *Neuron,* 38**,** 819-29.

CONTESTABILE, A. 2011. The history of the cholinergic hypothesis. *Behav Brain Res,* 221**,** 334-40.

CONTESTABILE, A., FILA, T., BARTESAGHI, R., CONTESTABILE, A. & CIANI, E. 2006. Choline acetyltransferase activity at different ages in brain of Ts65Dn mice, an animal model for Down's syndrome and related neurodegenerative diseases. *Journal of Neurochemistry,* 97**,** 515-526.

COOPER, J. D., SALEHI, A., DELCROIX, J. D., HOWE, C. L., BELICHENKO, P. V., CHUA-COUZENS, J., KILBRIDGE, J. F., CARLSON, E. J., EPSTEIN, C. J. & MOBLEY, W. C. 2001. Failed retrograde transport of NGF in a mouse model of Down's syndrome: reversal of cholinergic neurodegenerative phenotypes following NGF infusion. *Proc Natl Acad Sci U S A,* 98**,** 10439-44.

COPPOLA, J. J. & DISNEY, A. A. 2018. Is There a Canonical Cortical Circuit for the Cholinergic System? Anatomical Differences Across Common Model Systems. *Frontiers in Neural Circuits,* 12.

COSKUN, P. E. & BUSCIGLIO, J. 2012. Oxidative Stress and Mitochondrial Dysfunction in Down's Syndrome: Relevance to Aging and Dementia. *Current Gerontology and Geriatrics Research,* 2012**,** 383170.

COYLE, J. T., OSTER-GRANITE, M. L. & GEARHART, J. D. 1986. The neurobiologic consequences of Down syndrome. *Brain Res.Bull.,* 16**,** 773-787.

COYLE, J. T., OSTER-GRANITE, M. L., REEVES, R., HOHMANN, C., CORSI, P. & GEARHART, J. 1991. Down syndrome and the trisomy 16 mouse: impact of gene imbalance on brain development and aging. *Res.Publ.Assoc.Res.Nerv.Ment.Dis.,* 69**,** 85-99.

COYLE, J. T., OSTER-GRANITE, M. L., REEVES, R. H. & GEARHART, J. D. 1988. Down syndrome, Alzheimer's disease and the trisomy 16 mouse. *Trends in Neurosciences,* 11**,** 390-394.

COYLE, J. T., PRICE, D. L. & DELONG, M. R. 1983. Alzheimer's disease: a disorder of cortical cholinergic innervation. *Science,* 219**,** 1184-90.

DAVIDSON, K. C., JAMSHIDI, P., DALY, R., HEARN, M. T., PERA, M. F. & DOTTORI, M. 2007. Wnt3a regulates survival, expansion, and maintenance of neural progenitors derived from human embryonic stem cells. *Mol Cell Neurosci,* 36**,** 408-15.

DAVIES, P. & MALONEY, A. J. 1976. Selective loss of central cholinergic neurons in Alzheimer's disease. *Lancet,* 2**,** 1403.

DAVISSON, M. T., SCHMIDT, C., REEVES, R. H., IRVING, N. G., AKESON, E. C., HARRIS, B. S. & BRONSON, R. T. 1993. Segmental trisomy as a mouse model for Down syndrome. *Prog Clin Biol Res,* 384**,** 117-33.

DE CARLOS, J. A., SCHLAGGAR, B. L. & O'LEARY, D. D. M. 1995. Development of acetylcholinesterase-positive thalamic and basal forebrain afferents to embryonic rat neocortex. *Experimental Brain Research,* 104**,** 385-401.

DE GRAAF, G., BUCKLEY, F. & SKOTKO, B. G. 2015. Estimates of the Live Births, Natural Losses, and Elective Terminations with Down Syndrome in the United States. *American Journal of Medical Genetics Part A,* 167**,** 756-767.

DE SOUZA SILVA, M. A., DOLGA, A., PIERI, I., MARCHETTI, L., EISEL, U. L., HUSTON, J. P. & DERE, E. 2006. Cholinergic cells in the nucleus basalis of mice express the N-methyl-D-aspartate-receptor subunit NR2C and its replacement by the NR2B subunit enhances frontal and amygdaloid acetylcholine levels. *Genes Brain Behav,* 5**,** 552-60.

DELAUNAY, D., CORTAY, V., PATTI, D., KNOBLAUCH, K. & DEHAY, C. 2014. Mitotic spindle asymmetry: a Wnt/PCP-regulated mechanism generating asymmetrical division in cortical precursors. *Cell Rep,* 6**,** 400-14.

DÉTÁRI, L. 2000. Tonic and phasic influence of basal forebrain unit activity on the cortical EEG. *Behav Brain Res,* 115**,** 159-70.

DOBIN, A., DAVIS, C. A., SCHLESINGER, F., DRENKOW, J., ZALESKI, C., JHA, S., BATUT, P., CHAISSON, M. & GINGERAS, T. R. 2013. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics,* 29**,** 15-21.

DRAVID, G., YE, Z., HAMMOND, H., CHEN, G., PYLE, A., DONOVAN, P., YU, X. & CHENG, L. 2005. Defining the role of Wnt/beta-catenin signaling in the survival, proliferation, and self-renewal of human embryonic stem cells. *Stem Cells,* 23**,** 1489-501.

DU, T., XU, Q., OCBINA, P. J. & ANDERSON, S. A. 2008. NKX2.1 specifies cortical interneuron fate by activating Lhx6. *Development,* 135**,** 1559-67.

DU, X. G., WANG, X. Y. & GENG, M. Y. 2018. Alzheimer's disease hypothesis and related therapies. *Translational Neurodegeneration,* 7**,** 7.

DU, Z. W., CHEN, H., LIU, H., LU, J., QIAN, K., HUANG, C. L., ZHONG, X., FAN, F. & ZHANG, S. C. 2015. Generation and expansion of highly pure motor neuron progenitors from human pluripotent stem cells. *Nat Commun,* 6**,** 6626.

DUAN, L., BHATTACHARYYA, B. J., BELMADANI, A., PAN, L., MILLER, R. J. & KESSLER, J. A. 2014. Stem cell derived basal forebrain cholinergic neurons from Alzheimer's disease patients are more susceptible to cell death. *Mol Neurodegener,* 9**,** 3.

DUBOIS, B., RUBERG, M., JAVOY-AGID, F., PLOSKA, A. & AGID, Y. 1983. A subcortico-cortical cholinergic system is affected in Parkinson's disease. *Brain Res,* 288**,** 213-8.

DUCHON, A., RAVEAU, M., CHEVALIER, C., NALESSO, V., SHARP, A. J. & HERAULT, Y. 2011. Identification of the translocation breakpoints in the Ts65Dn and Ts1Cje mouse lines: relevance for modeling down syndrome. *Mammalian Genome,* 22**,** 674-684.

DUMAS, J. A. & NEWHOUSE, P. A. 2011. The cholinergic hypothesis of cognitive aging revisited again: cholinergic functional compensation. *Pharmacol.Biochem.Behav.,* 99**,** 254-261.

DUNCAN, G. W. 2011. The Aging Brain and Neurodegenerative Diseases. *Clinics in Geriatric Medicine,* 27**,** 629-+.

EADY, N., SHEEHAN, R., RANTELL, K., SINAI, A., BERNAL, J., BOHNEN, I., BONELL, S., COURTENAY, K., DODD, K., GAZIZOVA, D., HASSIOTIS, A., HILLIER, R., MCBRIEN, J., MUKHERJI, K., NAEEM, A., PEREZ-ACHIAGA, N., SHARMA, V., THOMAS, D., WALKER, Z., MCCARTHY, J. & STRYDOM, A. 2018. Impact of cholinesterase inhibitors or memantine on survival in adults with Down syndrome and dementia: clinical cohort study. *Br J Psychiatry,* 212**,** 155-160.

EICKHOFF, S., FRANZEN, L., KORDA, A., ROGG, H., TRULLEY, V. N., BORGWARDT, S. & AVRAM, M. 2022. The Basal Forebrain Cholinergic Nuclei and Their Relevance to Schizophrenia and Other Psychotic Disorders. *Front Psychiatry,* 13**,** 909961.

ELSHATORY, Y. & GAN, L. 2008. The LIM-homeobox gene Islet-1 is required for the development of restricted forebrain cholinergic neurons. *J Neurosci,* 28**,** 3291-7.

ENDO, R., CHEN, Y. K., BURKE, J., TAKASHIMA, N., SURYAWANSHI, N., HUI, K. K., MIYAZAKI, T. & TANAKA, M. 2023. Dysregulation of ribosome-associated quality control elicits cognitive disorders via overaccumulation of TTC3. *Proc Natl Acad Sci U S A,* 120**,** e2211522120.

FAHNESTOCK, M. & SHEKARI, A. 2019. ProNGF and Neurodegeneration in Alzheimer's Disease. *Frontiers in Neuroscience,* 13.

FATHI, A., MATHIVANAN, S., KONG, L., PETERSEN, A. J., HARDER, C. R. K., BLOCK, J., MILLER, J. M., BHATTACHARYYA, A., WANG, D. & ZHANG, S. C. 2022. Chemically induced senescence in human stem cell-derived neurons promotes phenotypic presentation of neurodegeneration. *Aging Cell,* 21**,** e13541.

FERNANDEZ, M. M. & LOPEZ, V. M. 2020. Simultaneous auditory-visual support in grammatical intervention in subjects with intellectual disability. *Revista De Educacion***,** 115-140.

FERNANDEZ-CABELLO, S., KRONBICHLER, M., VAN DIJK, K. R. A., GOODMAN, J. A., SPRENG, R. N. & SCHMITZ, T. W. 2020. Basal forebrain volume reliably predicts the cortical spread of Alzheimer's degeneraion. 143**,** 1009.

FERRANTE, R. J., BEAL, M. F., KOWALL, N. W., RICHARDSON, E. P., JR. & MARTIN, J. B. 1987. Sparing of acetylcholinesterase-containing striatal neurons in Huntington's disease. *Brain Res,* 411**,** 162-6.

FERREIRA-VIEIRA, T. H., GUIMARAES, I. M., SILVA, F. R. & RIBEIRO, F. M. 2016. Alzheimer's Disease: Targeting the Cholinergic System. *Current Neuropharmacology,* 14**,** 101-115.

FINESILVER, C. 2002. A new age for childhood diseases. Down syndrome. *Rn,* 65**,** 43-8; quiz 49.

FOLCH, J., BUSQUETS, O., ETTCHETO, M., SÁNCHEZ-LÓPEZ, E., CASTRO-TORRES, R. D., VERDAGUER, E., GARCIA, M. L., OLLOQUEQUI, J., CASADESÚS, G. & BEAS-ZARATE, C. 2018. Memantine for the treatment of dementia: a review on its current and future applications. *Journal of Alzheimer's Disease,* 62**,** 1223-1240.

FORTEA, J., VILAPLANA, E., CARMONA-IRAGUI, M., BENEJAM, B., VIDELA, L., BARROETA, I., FERNÁNDEZ, S., ALTUNA, M., PEGUEROLES, J., MONTAL, V., VALLDENEU, S., GIMÉNEZ, S., GONZÁLEZ-ORTIZ, S., MUÑOZ, L., ESTELLÉS, T., ILLÁN-GALA, I., BELBIN, O., CAMACHO, V., WILSON, L. R., ANNUS, T., OSORIO, R. S., VIDELA, S., LEHMANN, S., HOLLAND, A. J., ALCOLEA, D., CLARIMÓN, J., ZAMAN, S. H., BLESA, R. & LLEÓ, A. 2020. Clinical and biomarker changes of Alzheimer's disease in adults with Down syndrome: a cross-sectional study. *The Lancet,* 395**,** 1988-1997.

FORTEA, J., ZAMAN, S. H., HARTLEY, S., RAFII, M. S., HEAD, E. & CARMONA-IRAGUI, M. 2021. Alzheimer's disease associated with Down syndrome: a genetic form of dementia. *The Lancet Neurology,* 20**,** 930-942.

FRAGKOULI, A., HEARN, C., ERRINGTON, M., COOKE, S., GRIGORIOU, M., BLISS, T., STYLIANOPOULOU, F. & PACHNIS, V. 2005. Loss of forebrain cholinergic neurons and impairment in spatial learning and memory in LHX7-deficient mice. *Eur J Neurosci,* 21**,** 2923-38.

FROST, B., JACKS, R. L. & DIAMOND, M. I. 2009. Propagation of Tau Misfolding from the Outside to the Inside of a Cell. *Journal of Biological Chemistry,* 284**,** 12845-12852.

FULDA, S. & DEBATIN, K. M. 2006. Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy. *Oncogene,* 25**,** 4798-811.

FURUSHO, M., ONO, K., TAKEBAYASHI, H., MASAHIRA, N., KAGAWA, T., IKEDA, K. & IKENAKA, K. 2006. Involvement of the Olig2 transcription factor in cholinergic neuron development of the basal forebrain. *Developmental Biology,* 293**,** 348-357.

GALLUZZI, L., BLOMGREN, K. & KROEMER, G. 2009. Mitochondrial membrane permeabilization in neuronal injury. *Nature Reviews Neuroscience,* 10**,** 481-494.

GALLUZZI, L., VITALE, I., AARONSON, S. A., ABRAMS, J. M., ADAM, D., AGOSTINIS, P., ALNEMRI, E. S., ALTUCCI, L., AMELIO, I., ANDREWS, D. W., ANNICCHIARICO-PETRUZZELLI, M., ANTONOV, A. V., ARAMA, E., BAEHRECKE, E. H., BARLEV, N. A., BAZAN, N. G., BERNASSOLA, F., BERTRAND, M. J. M., BIANCHI, K., BLAGOSKLONNY, M. V., BLOMGREN, K., BORNER, C., BOYA, P., BRENNER, C., CAMPANELLA, M., CANDI, E., CARMONA-GUTIERREZ, D., CECCONI, F., CHAN, F. K., CHANDEL, N. S., CHENG, E. H., CHIPUK, J. E., CIDLOWSKI, J. A., CIECHANOVER, A., COHEN, G. M., CONRAD, M., CUBILLOS-RUIZ, J. R., CZABOTAR, P. E., D'ANGIOLELLA, V., DAWSON, T. M., DAWSON, V. L., DE LAURENZI, V., DE MARIA, R., DEBATIN, K. M., DEBERARDINIS, R. J., DESHMUKH, M., DI DANIELE, N., DI VIRGILIO, F., DIXIT, V. M., DIXON, S. J., DUCKETT, C. S., DYNLACHT, B. D., EL-DEIRY, W. S., ELROD, J. W., FIMIA, G. M., FULDA, S., GARCÍA-SÁEZ, A. J., GARG, A. D., GARRIDO, C., GAVATHIOTIS, E., GOLSTEIN, P., GOTTLIEB, E., GREEN, D. R., GREENE, L. A., GRONEMEYER, H., GROSS, A., HAJNOCZKY, G., HARDWICK, J. M., HARRIS, I. S., HENGARTNER, M. O., HETZ, C., ICHIJO, H., JÄÄTTELÄ, M., JOSEPH, B., JOST, P. J., JUIN, P. P., KAISER, W. J., KARIN, M., KAUFMANN, T., KEPP, O., KIMCHI, A., KITSIS, R. N., KLIONSKY, D. J., KNIGHT, R. A., KUMAR, S., LEE, S. W., LEMASTERS, J. J., LEVINE, B., LINKERMANN, A., LIPTON, S. A., LOCKSHIN, R. A., LÓPEZ-OTÍN, C., LOWE, S. W., LUEDDE, T., LUGLI, E., MACFARLANE, M., MADEO, F., MALEWICZ, M., MALORNI, W., MANIC, G., et al. 2018. Molecular mechanisms of cell death: recommendations of the Nomenclature Committee on Cell Death 2018. *Cell Death Differ,* 25**,** 486-541.

GAMAGE, R., WAGNON, I., ROSSETTI, I., CHILDS, R., NIEDERMAYER, G., CHESWORTH, R. & GYENGESI, E. 2020. Cholinergic Modulation of Glial Function During Aging and Chronic Neuroinflammation. *Frontiers in Cellular Neuroscience,* 14.

GARDINER, K. & DAVISSON, M. 2000. The sequence of human chromosome 21 and implications for research into Down syndrome. *Genome Biol.,* 1**,** REVIEWS0002.

GARDINER, K., FORTNA, A., BECHTEL, L. & DAVISSON, M. T. 2003. Mouse models of Down syndrome: how useful can they be? Comparison of the gene content of human chromosome 21 with orthologous mouse genomic regions. *Gene,* 318**,** 137-147.

GEULA, C., DUNLOP, S. R., AYALA, I., KAWLES, A. S., FLANAGAN, M. E., GEFEN, T. & MESULAM, M. M. 2021. Basal forebrain cholinergic system in the dementias: Vulnerability, resilience, and resistance. *J Neurochem,* 158**,** 1394-1411.

GEULA, C., NAGYKERY, N., NICHOLAS, A. & WU, C.-K. 2008. Cholinergic neuronal and axonal abnormalities are present early in aging and in Alzheimer disease. *Journal of Neuropathology & Experimental Neurology,* 67**,** 309-318.

GIBBS, R. B., MCCABE, J. T., BUCK, C. R., CHAO, M. V. & PFAFF, D. W. 1989. Expression of NGF receptor in the rat forebrain detected with in situ hybridization and immunohistochemistry. *Molecular Brain Research,* 6**,** 275-287.

GIFFIN-RAO, Y., SHENG, J., STRAND, B., XU, K., HUANG, L., MEDO, M., RISGAARD, K. A., DANTINNE, S., MOHAN, S., KESHAN, A., DALEY, R. A., JR., LEVESQUE, B., AMUNDSON, L., REESE, R., SOUSA, A. M. M., TAO, Y., WANG, D., ZHANG, S. C. & BHATTACHARYYA, A. 2022. Altered patterning of trisomy 21 interneuron progenitors. *Stem Cell Reports,* 17**,** 1366-1379.

GINSBERG, S. D., CHE, S., WUU, J., COUNTS, S. E. & MUFSON, E. J. 2006. Down regulation of trk but not p75NTR gene expression in single cholinergic basal forebrain neurons mark the progression of Alzheimer's disease. *J Neurochem,* 97**,** 475-87.

GOARD, M. & DAN, Y. 2009. Basal forebrain activation enhances cortical coding of natural scenes. *Nature Neuroscience,* 12**,** 1444-1449.

GOEDERT, M., SPILLANTINI, M. G., JAKES, R., RUTHERFORD, D. & CROWTHER, R. A. 1989. MULTIPLE ISOFORMS OF HUMAN MICROTUBULE-ASSOCIATED PROTEIN-TAU - SEQUENCES AND LOCALIZATION IN NEUROFIBRILLARY TANGLES OF ALZHEIMERS-DISEASE. *Neuron,* 3**,** 519-526.

GOLDEN, J. A. & HYMAN, B. T. 1994. Development of the superior temporal neocortex is anomalous in trisomy 21. *J.Neuropathol.Exp.Neurol.,* 53**,** 513-520.

GOMEZ, W., MORALES, R., MARACAJA-COUTINHO, V., PARRA, V. & NASSIF, M. 2020. Down syndrome and Alzheimer's disease: common molecular traits beyond the amyloid precursor protein. *Aging-Us,* 12**,** 1011-1033.

GOUGH, G., O'BRIEN, N. L., ALIC, I., GOH, P. A., YEAP, Y. J., GROET, J., NIZETIC, D. & MURRAY, A. 2020. Modeling Down syndrome in cells: From stem cells to organoids. *Prog Brain Res,* 251**,** 55-90.

GRANNO, S., NIXON-ABELL, J., BERWICK, D. C., TOSH, J., HEATON, G., ALMUDIMEEGH, S., NAGDA, Z., RAIN, J. C., ZANDA, M., PLAGNOL, V., TYBULEWICZ, V. L. J., CLEVERLEY, K., WISEMAN, F. K., FISHER, E. M. C. & HARVEY, K. 2019. Downregulated Wnt/β-catenin signalling in the Down syndrome hippocampus. *Sci Rep,* 9**,** 7322.

GREEN, D. R. & LLAMBI, F. 2015. Cell Death Signaling. *Cold Spring Harb Perspect Biol,* 7.

GROTHE, M. J., SEPULCRE, J., GONZALEZ-ESCAMILLA, G., JELISTRATOVA, I., SCHÖLL, M., HANSSON, O., TEIPEL, S. J. & INITIATIVE, A. S. D. N. 2018. Molecular properties underlying regional vulnerability to Alzheimer's disease pathology. *Brain,* 141**,** 2755-2771.

GRUNDKE-IQBAL, I., IQBAL, K., TUNG, Y. C., QUINLAN, M., WISNIEWSKI, H. M. & BINDER, L. I. 1986. Abnormal phosphorylation of the microtubule-associated protein tau (tau) in Alzheimer cytoskeletal pathology. *Proc.Natl.Acad.Sci.U.S.A,* 83**,** 4913-4917.

GU, L. & GUO, Z. 2013. Alzheimer's Aβ42 and Aβ40 peptides form interlaced amyloid fibrils. *J Neurochem,* 126**,** 305-11.

GUIDI, S., BONASONI, P., CECCARELLI, C., SANTINI, D., GUALTIERI, F., CIANI, E. & BARTESAGHI, R. 2008. Neurogenesis impairment and increased cell death reduce total neuron number in the hippocampal region of fetuses with Down syndrome. *Brain Pathol,* 18**,** 180-97.

GUIDI, S., CIANI, E., BONASONI, P., SANTINI, D. & BARTESAGHI, R. 2011. Widespread proliferation impairment and hypocellularity in the cerebellum of fetuses with down syndrome. *Brain Pathol.,* 21**,** 361-373.

GUIDI, S., GIACOMINI, A., STAGNI, F., EMILI, M., UGUAGLIATI, B., BONASONI, M. P. & BARTESAGHI, R. 2018. ABNORMAL DEVELOPMENT OF THE INFERIOR TEMPORAL REGION IN FETUSES WITH DOWN SYNDROME. *Brain Pathol.*

GUIDI, S., STAGNI, F., BIANCHI, P., CIANI, E., RAGAZZI, E., TRAZZI, S., GROSSI, G., MANGANO, C., CALZA, L. & BARTESAGHI, R. 2013. Early Pharmacotherapy with Fluoxetine Rescues Dendritic Pathology in the Ts65Dn Mouse Model of Down Syndrome. *Brain Pathology,* 23**,** 129-143.

GUIHARD-COSTA, A. M., KHUNG, S., DELBECQUE, K., MÉNEZ, F. & DELEZOIDE, A. L. 2006. Biometry of face and brain in fetuses with trisomy 21. *Pediatr Res,* 59**,** 33-8.

GULACSI, A. & ANDERSON, S. A. 2006. Shh maintains Nkx2.1 in the MGE by a Gli3-independent mechanism. *Cereb Cortex,* 16 Suppl 1**,** i89-95.

GURSES, M. S., URAL, M. N., GULEC, M. A., AKYOL, O. & AKYOL, S. 2016. Pathophysiological function of ADAMTS enzymes on molecular mechanism of Alzheimer's disease. *Aging and disease,* 7**,** 479.

HAMERTON, J. L., GIANNELLI, F. & POLANI, P. E. 1965. CYTOGENETICS OF DOWN'S SYNDROME (MONGOLISM). I. DATA ON A CONSECUTIVE SERIES OF PATIENTS REFERRED FOR GENETIC COUNSELLING AND DIAGNOSIS. *Cytogenetics,* 4**,** 171-85.

HAMLETT, E. D., BOGER, H. A., LEDREUX, A., KELLEY, C. M., MUFSON, E. J., FALANGOLA, M. F., GUILFOYLE, D. N., NIXON, R. A., PATTERSON, D., DUVAL, N. & GRANHOLM, A. C. E. 2016. Cognitive Impairment, Neuroimaging, and Alzheimer Neuropathology in Mouse Models of Down Syndrome. *Current Alzheimer Research,* 13**,** 35-52.

HANDEN, B. L., COHEN, A. D., CHANNAMALAPPA, U., BULOVA, P., CANNON, S. A., COHEN, W. I., MATHIS, C. A., PRICE, J. C. & KLUNK, W. E. 2012. Imaging brain amyloid in nondemented young adults with Down syndrome using Pittsburgh compound B. *Alzheimers & Dementia,* 8**,** 496-501.

HANDEN, B. L., LOTT, I. T., CHRISTIAN, B. T., SCHUPF, N., OBRYANT, S., MAPSTONE, M., FAGAN, A. M., LEE, J. H., TUDORASCU, D. & WANG, M. C. 2020. The Alzheimer's Biomarker Consortium-Down Syndrome: Rationale and methodology. *Alzheimer's & Dementia: Diagnosis, Assessment & Disease Monitoring,* 12**,** e12065.

HANSEEUW, B. J., BETENSKY, R. A., JACOBS, H. I., SCHULTZ, A. P., SEPULCRE, J., BECKER, J. A., COSIO, D. M. O., FARRELL, M., QUIROZ, Y. T. & MORMINO, E. C. 2019. Association of amyloid and tau with cognition in preclinical Alzheimer disease: a longitudinal study. *JAMA neurology,* 76**,** 915-924.

HANSSON, O., LEHMANN, S., OTTO, M., ZETTERBERG, H. & LEWCZUK, P. 2019. Advantages and disadvantages of the use of the CSF Amyloid β (Aβ) 42/40 ratio in the diagnosis of Alzheimer's Disease. *Alzheimer's research & therapy* [Online], 11. Available: http://europepmc.org/abstract/MED/31010420
https://alzres.biomedcentral.com/track/pdf/10.1186/s13195-019-0485-0
https://doi.org/10.1186/s13195-019-0485-0
https://europepmc.org/articles/PMC6477717
https://europepmc.org/articles/PMC6477717?pdf=render [Accessed 2019/04//].

HAO, Y., STUART, T., KOWALSKI, M. H., CHOUDHARY, S., HOFFMAN, P., HARTMAN, A., SRIVASTAVA, A., MOLLA, G., MADAD, S., FERNANDEZ-GRANDA, C. & SATIJA, R. 2023. Dictionary learning for integrative, multimodal and scalable single-cell analysis. *Nature Biotechnology*.

HARDY, J. & ALLSOP, D. 1991. Amyloid deposition as the central event in the aetiology of Alzheimer's disease. *Trends Pharmacol.Sci.,* 12**,** 383-388.

HARKANY, T., HÄRTIG, W., BERGHUIS, P., DOBSZAY, M. B., ZILBERTER, Y., EDWARDS, R. H., MACKIE, K. & ERNFORS, P. 2003. Complementary distribution of type 1 cannabinoid receptors and vesicular glutamate transporter 3 in basal forebrain suggests input-specific retrograde signalling by cholinergic neurons. *Eur J Neurosci,* 18**,** 1979-92.

HARTLEY, S. L., HANDEN, B. L., DEVENNY, D., TUDORASCU, D., PIRO-GAMBETTI, B., ZAMMIT, M. D., LAYMON, C. M., KLUNK, W. E., ZAMAN, S. & COHEN, A. 2020. Cognitive indicators of transition to preclinical and prodromal stages of Alzheimer's disease in Down syndrome. *Alzheimer's & Dementia: Diagnosis, Assessment & Disease Monitoring,* 12**,** e12096.

HASSELMO, M. E. 2006. The role of acetylcholine in learning and memory. *Current opinion in neurobiology,* 16**,** 710-715.

HATTORI, M., FUJIYAMA, A., TAYLOR, T. D., WATANABE, H., YADA, T., PARK, H. S., TOYODA, A., ISHII, K., TOTOKI, Y., CHOI, D. K., SOEDA, E., OHKI, M., TAKAGI, T., SAKAKI, Y., TAUDIEN, S., BLECHSCHMIDT, K., POLLEY, A., MENZEL, U., DELABAR, J., KUMPF, K., LEHMANN, R., PATTERSON, D., REICHWALD, K., RUMP, A., SCHILLHABEL, M., SCHUDY, A., ZIMMERMANN, W., ROSENTHAL, A., KUDOH, J., SCHIBUYA, K., KAWASAKI, K., ASAKAWA, S., SHINTANI, A., SASAKI, T., NAGAMINE, K., MITSUYAMA, S., ANTONARAKIS, S. E., MINOSHIMA, S., SHIMIZU, N., NORDSIEK, G., HORNISCHER, K., BRANT, P., SCHARFE, M., SCHON, O., DESARIO, A., REICHELT, J., KAUER, G., BLOCKER, H., RAMSER, J., BECK, A., KLAGES, S., HENNIG, S., RIESSELMANN, L., DAGAND, E., HAAF, T., WEHRMEYER, S., BORZYM, K., GARDINER, K., NIZETIC, D., FRANCIS, F., LEHRACH, H., REINHARDT, R. & YASPO, M. L. 2000. The DNA sequence of human chromosome 21. *Nature,* 405**,** 311-319.

HE, J., LIN, L. & CHEN, J. 2022. Practical bioinformatics pipelines for single-cell RNA-seq data analysis. *Biophys Rep,* 8**,** 158-169.

HEAD, E., LOTT, I. T., WILCOCK, D. M. & LEMERE, C. A. 2016. Aging in Down Syndrome and the Development of Alzheimer's Disease Neuropathology. *Current Alzheimer Research,* 13**,** 18-29.

HEAD, E., POWELL, D., GOLD, B. T. & SCHMITT, F. A. 2012. Alzheimer's Disease in Down Syndrome. *Eur J Neurodegener Dis,* 1**,** 353-364.

HEDREEN, J. C., STRUBLE, R. G., WHITEHOUSE, P. J. & PRICE, D. L. 1984. Topography of the magnocellular basal forebrain system in human brain. *J Neuropathol Exp Neurol,* 43**,** 1-21.

HEEG-TRUESDELL, E. & LABONNE, C. 2006. Neural induction in Xenopus requires inhibition of Wnt-beta-catenin signaling. *Dev Biol,* 298**,** 71-86.

HENEKA, M. T., KUMMER, M. P., STUTZ, A., DELEKATE, A., SCHWARTZ, S., VIEIRA-SAECKER, A., GRIEP, A., AXT, D., REMUS, A., TZENG, T. C., GELPI, E., HALLE,

A., KORTE, M., LATZ, E. & GOLENBOCK, D. T. 2013. NLRP3 is activated in Alzheimer's disease and contributes to pathology in APP/PS1 mice. *Nature,* 493**,** 674-8.

HERAULT, Y., DELABAR, J. M., FISHER, E. M. C., TYBULEWICZ, V. L. J., YU, E. & BRAULT, V. 2017. Rodent models in Down syndrome research: impact and future opportunities. *Disease Models & Mechanisms,* 10**,** 1165-1186.

HERGENREDER, E., ZORINA, Y., ZHAO, Z., MUNGUBA, H., CALDER, E. L., BAGGIOLINI, A., MINOTTI, A. P., WALSH, R. M., LISTON, C., LEVITZ, J., GARIPPA, R., CHEN, S., CICERI, G. & STUDER, L. 2022. Combined small molecule treatment accelerates timing of maturation in human pluripotent stem cell-derived neurons. *bioRxiv***,** 2022.06.02.494616.

HEYS, J. G., GIOCOMO, L. M. & HASSELMO, M. E. 2010. Cholinergic modulation of the resonance properties of stellate cells in layer II of medial entorhinal cortex. *Journal of Neurophysiology,* 104**,** 258-270.

HIBAOUI, Y., GRAD, I., LETOURNEAU, A., SAILANI, M. R., DAHOUN, S., SANTONI, F. A., GIMELLI, S., GUIPPONI, M., PELTE, M. F., BENA, F., ANTONARAKIS, S. E. & FEKI, A. 2014. Modelling and rescuing neurodevelopmental defect of Down syndrome using induced pluripotent stem cells from monozygotic twins discordant for trisomy 21. *Embo Molecular Medicine,* 6**,** 259-277.

HITHERSAY, R., STARTIN, C. M., HAMBURG, S., MOK, K. Y., HARDY, J., FISHER, E. M. C., TYBULEWICZ, V. L. J., NIZETIC, D. & STRYDOM, A. 2019. Association of Dementia With Mortality Among Adults With Down Syndrome Older Than 35 Years. *JAMA Neurol,* 76**,** 152-160.

HOLLAND, A. J., HON, J., HUPPERT, F. A. & STEVENS, F. 2000. Incidence and course of dementia in people with Down's syndrome: findings from a population-based study. *J.Intellect.Disabil.Res.,* 44 (Pt 2)**,** 138-146.

HOOLI, B., MOHAPATRA, G., MATTHEISEN, M., PARRADO, A., ROEHR, J., SHEN, Y., GUSELLA, J., MOIR, R., SAUNDERS, A. & LANGE, C. 2012. Role of common and rare APP DNA sequence variants in Alzheimer disease. *Neurology,* 78**,** 1250-1257.

HU, B.-Y., WEICK, J. P., YU, J., MA, L.-X., ZHANG, X.-Q., THOMSON, J. A. & ZHANG, S.-C. 2010. Neural differentiation of human induced pluripotent stem cells follows developmental principles but with variable potency. *Proceedings of the National Academy of Sciences,* 107**,** 4335-4340.

HU, Y., QU, Z. Y., CAO, S. Y., LI, Q., MA, L., KRENCIK, R., XU, M. & LIU, Y. 2016. Directed differentiation of basal forebrain cholinergic neurons from human pluripotent stem cells. *J Neurosci Methods,* 266**,** 42-9.

HUANG, C. T., TAO, Y., LU, J., JONES, J. R., FOWLER, L., WEICK, J. P. & ZHANG, S. C. 2016. Time-Course Gene Expression Profiling Reveals a Novel Role of Non-Canonical WNT Signaling During Neural Induction. *Sci Rep,* 6**,** 32600.

HUNTER, C. L., BACHMAN, D. & GRANHOLM, A. C. 2004. Minocycline prevents cholinergic loss in a mouse model of Down's syndrome. *Ann Neurol,* 56**,** 675-88.

HUO, H. Q., QU, Z. Y., YUAN, F., MA, L., YAO, L., XU, M., HU, Y., JI, J., BHATTACHARYYA, A., ZHANG, S. C. & LIU, Y. 2018a. Modeling Down Syndrome with Patient iPSCs Reveals Cellular and Migration Deficits of GABAergic Neurons. *Stem Cell Reports,* 10**,** 1251-1266.

HUO, H. Q., QU, Z. Y., YUAN, F., MA, L. X., YAO, L., XU, M., HU, Y., JI, J., BHATTACHARYYA, A., ZHANG, S. C. & LIU, Y. 2018b. Modeling Down Syndrome

with Patient iPSCs Reveals Cellular and Migration Deficits of GABAergic Neurons. *Stem Cell Reports,* 10**,** 1251-1266.

ISACSON, O., SEO, H., LIN, L., ALBECK, D. & GRANHOLM, A. C. 2002. Alzheimer's disease and Down's syndrome: roles of APP, trophic factors and ACh. 25**,** 84.

IWATA, R. & VANDERHAEGHEN, P. 2021. Regulatory roles of mitochondria and metabolism in neurogenesis. *Curr Opin Neurobiol,* 69**,** 231-240.

JACK JR, C. R., BENNETT, D. A., BLENNOW, K., CARRILLO, M. C., DUNN, B., HAEBERLEIN, S. B., HOLTZMAN, D. M., JAGUST, W., JESSEN, F. & KARLAWISH, J. 2018. NIA-AA research framework: toward a biological definition of Alzheimer's disease. *Alzheimer's & Dementia,* 14**,** 535-562.

JEONG, J. H., WOO, Y. J., CHUA, S., JR. & JO, Y. H. 2016. Single-Cell Gene Expression Analysis of Cholinergic Neurons in the Arcuate Nucleus of the Hypothalamus. *PLoS One,* 11**,** e0162839.

JERBER, J., HALDANE, J., STEER, J., PEARCE, D. & PATEL, M. 2020. Dissociation of neuronal culture to single cells for scRNA-seq (10x Genomics)

JIANG, J., JING, Y. C., COST, G. J., CHIANG, J. C., KOLPA, H. J., COTTON, A. M., CARONE, D. M., CARONE, B. R., SHIVAK, D. A., GUSCHIN, D. Y., PEARL, J. R., REBAR, E. J., BYRON, M., GREGORY, P. D., BROWN, C. J., URNOV, F. D., HALL, L. L. & LAWRENCE, J. B. 2013. Translating dosage compensation to trisomy 21. *Nature,* 500**,** 296-+.

JONES, B. E. 2008. Modulation of cortical activation and behavioral arousal by cholinergic and orexinergic systems. *Ann N Y Acad Sci,* 1129**,** 26-34.

JOVANOVIC, S. V., CLEMENTS, D. & MACLEOD, K. 1998. Biomarkers of oxidative stress are significantly elevated in Down syndrome. *Free Radical Biology and Medicine,* 25**,** 1044-1048.

KAMIYA, D., BANNO, S., SASAI, N., OHGUSHI, M., INOMATA, H., WATANABE, K., KAWADA, M., YAKURA, R., KIYONARI, H., NAKAO, K., JAKT, L. M., NISHIKAWA, S. & SASAI, Y. 2011. Intrinsic transition of embryonic stem-cell differentiation into neural progenitors. *Nature,* 470**,** 503-9.

KASHIRINA, A., GAVRINA, A., KRYUKOV, E., ELAGIN, V., KOLESOVA, Y., ARTYUHOV, A., MOMOTYUK, E., ABDYYEV, V., MESHCHERYAKOVA, N., ZAGAYNOVA, E., DASHINIMAEV, E. & KASHINA, A. 2021. Energy Metabolism and Intracellular pH Alteration in Neural Spheroids Carrying Down Syndrome. *Biomedicines,* 9.

KASUGA, K., SHIMOHATA, T., NISHIMURA, A., SHIGA, A., MIZUGUCHI, T., TOKUNAGA, J., OHNO, T., MIYASHITA, A., KUWANO, R. & MATSUMOTO, N. 2009. Identification of independent APP locus duplication in Japanese patients with early-onset Alzheimer disease. *Journal of Neurology, Neurosurgery & Psychiatry,* 80**,** 1050-1052.

KATO, T. 1989. Choline acetyltransferase activities in single spinal motor neurons from patients with amyotrophic lateral sclerosis. *J Neurochem,* 52**,** 636-40.

KAUR, S., JUNEK, A., BLACK, M. A. & SEMBA, K. 2008. Effects of ibotenate and 192IgG-saporin lesions of the nucleus basalis magnocellularis/substantia innominata on spontaneous sleep and wake states and on recovery sleep after sleep deprivation in rats. *J Neurosci,* 28**,** 491-504.

KELLEY, C. M., GINSBERG, S. D., ALLDRED, M. J., STRUPP, B. J. & MUFSON, E. J. 2019. Maternal Choline Supplementation Alters Basal Forebrain Cholinergic Neuron Gene Expression in the Ts65Dn Mouse Model of Down Syndrome. *Dev Neurobiol*.

KELLEY, C. M., POWERS, B. E., VELAZQUEZ, R., ASH, J. A., GINSBERG, S. D., STRUPP, B. J. & MUFSON, E. J. 2014a. Maternal choline supplementation differentially alters the basal forebrain cholinergic system of young-adult Ts65Dn and disomic mice. *J Comp Neurol,* 522**,** 1390-410.

KELLEY, C. M., POWERS, B. E., VELAZQUEZ, R., ASH, J. A., GINSBERG, S. D., STRUPP, B. J. & MUFSON, E. J. 2014b. Sex Differences in the Cholinergic Basal Forebrain in the Ts65Dn Mouse Model of Down Syndrome and Alzheimer's Disease. *Brain Pathology,* 24**,** 33-44.

KHAN, U. A., LIU, L., PROVENZANO, F. A., BERMAN, D. E., PROFACI, C. P., SLOAN, R., MAYEUX, R., DUFF, K. E. & SMALL, S. A. 2014. Molecular drivers and cortical spread of lateral entorhinal cortex dysfunction in preclinical Alzheimer's disease. *Nature neuroscience,* 17**,** 304-311.

KIM, C. 2015. iPSC technology--Powerful hand for disease modeling and therapeutic screen. *BMB Rep,* 48**,** 256-65.

KIM, K., DOI, A., WEN, B., NG, K., ZHAO, R., CAHAN, P., KIM, J., ARYEE, M. J., JI, H., EHRLICH, L. I., YABUUCHI, A., TAKEUCHI, A., CUNNIFF, K. C., HONGGUANG, H., MCKINNEY-FREEMAN, S., NAVEIRAS, O., YOON, T. J., IRIZARRY, R. A., JUNG, N., SEITA, J., HANNA, J., MURAKAMI, P., JAENISCH, R., WEISSLEDER, R., ORKIN, S. H., WEISSMAN, I. L., FEINBERG, A. P. & DALEY, G. Q. 2010. Epigenetic memory in induced pluripotent stem cells. *Nature,* 467**,** 285-90.

KIM, Y.-J., TARASHANSKY, A., LIANG, K., URISKO, M., DORMAN, L., BORJA, M., NEFF, N., PISCO, A. O. & GRANADOS, A. 2023. Tutorial: guidelines for manual cell type annotation of single-cell multi-omics datasets using interactive software. *bioRxiv***,** 2023.07.11.548639.

KINNEY, J. W., BEMILLER, S. M., MURTISHAW, A. S., LEISGANG, A. M., SALAZAR, A. M. & LAMB, B. T. 2018. Inflammation as a central mechanism in Alzheimer's disease. *Alzheimer's & Dementia: Translational Research & Clinical Interventions,* 4**,** 575-590.

KISHNANI, P. S., SPIRIDIGLIOZZI, G. A., HELLER, J. H., SULLIVAN, J. A., DORAISWAMY, P. M. & KRISHNAN, K. R. 2001. Donepezil for Down's syndrome. *Am.J.Psychiatry,* 158**,** 143.

KISS, J., SCHLUMPF, M. & BALAZS, R. 1989. Selective retardation of the development of the basal forebrain cholinergic and pontine catecholaminergic nuclei in the brain of trisomy 16 mouse, an animal model of Down's syndrome. *Brain Res.Dev.Brain Res.,* 50**,** 251-264.

KITAZONO, I., HAMADA, T., YOSHIMURA, T., KIRISHIMA, M., YOKOYAMA, S., AKAHANE, T. & TANIMOTO, A. 2020. PCP4/PEP19 downregulates neurite outgrowth via transcriptional regulation of Ascl1 and NeuroD1 expression in human neuroblastoma M17 cells. *Lab Invest,* 100**,** 1551-1563.

KLUNK, W. E., PRICE, J. C., MATHIS, C. A., TSOPELAS, N. D., LOPRESTI, B. J., ZIOLKO, S. K., BI, W., HOGE, J. A., COHEN, A. D. & IKONOMOVIC, M. D. 2007. Amyloid deposition begins in the striatum of presenilin-1 mutation carriers from two unrelated pedigrees. *Journal of Neuroscience,* 27**,** 6174-6184.

KOELLIKER, A. 1896. *Handbuch der Gewebelehre des Menschen. In: Nervensystem des Menschen und der Thiere. vol 2, 6th edn. W. Engelmann, Leipzig*

KORBEL, J. O., TIROSH-WAGNER, T., URBAN, A. E., CHEN, X. N., KASOWSKI, M., DAI, L., GRUBERT, F., ERDMAN, C., GAO, M. C., LANGE, K., SOBEL, E. M., BARLOW, G. M., AYLSWORTH, A. S., CARPENTER, N. J., CLARK, R. D., COHEN, M. Y., DORAN, E., FALIK-ZACCAI, T., LEWIN, S. O., LOTT, I. T., MCGILLIVRAY, B. C., MOESCHLER, J. B., PETTENATI, M. J., PUESCHEL, S. M., RAO, K. W., SHAFFER, L. G., SHOHAT, M., VAN RIPER, A. J., WARBURTON, D., WEISSMAN, S., GERSTEIN, M. B., SNYDER, M. & KORENBERG, J. R. 2009. The genetic architecture of Down syndrome phenotypes revealed by high-resolution analysis of human segmental trisomies. *Proceedings of the National Academy of Sciences of the United States of America,* 106**,** 12031-12036.

KOSIK, K. S., JOACHIM, C. L. & SELKOE, D. J. 1986. Microtubule-associated protein tau (tau) is a major antigenic component of paired helical filaments in Alzheimer disease. *Proc Natl Acad Sci U S A,* 83**,** 4044-8.

KRAJKA, V., NAUJOCK, M., PAULY, M. G., STENGEL, F., MEIER, B., STANSLOWSKY, N., KLEIN, C., SEIBLER, P., WEGNER, F. & CAPETIAN, P. 2021. Ventral Telencephalic Patterning Protocols for Induced Pluripotent Stem Cells. *Front Cell Dev Biol,* 9**,** 716249.

KULESHOV, M. V., JONES, M. R., ROUILLARD, A. D., FERNANDEZ, N. F., DUAN, Q., WANG, Z., KOPLEV, S., JENKINS, S. L., JAGODNIK, K. M., LACHMANN, A., MCDERMOTT, M. G., MONTEIRO, C. D., GUNDERSEN, G. W. & MA'AYAN, A. 2016. Enrichr: a comprehensive gene set enrichment analysis web server 2016 update. *Nucleic Acids Res,* 44**,** W90-7.

LAKE, B. B. & SOKOL, S. Y. 2009. Strabismus regulates asymmetric cell divisions and cell fate determination in the mouse brain. *J Cell Biol,* 185**,** 59-66.

LANZILLOTTA, C. & DI DOMENICO, F. 2021. Stress Responses in Down Syndrome Neurodegeneration: State of the Art and Therapeutic Molecules. *Biomolecules,* 11.

LAO, P. J., HANDEN, B. L., BETTHAUSER, T. J., MIHAILA, I., HARTLEY, S. L., COHEN, A. D., TUDORASCU, D. L., BULOVA, P. D., LOPRESTI, B. J. & TUMULURU, R. V. 2017. Longitudinal changes in amyloid positron emission tomography and volumetric magnetic resonance imaging in the nondemented Down syndrome population. *Alzheimer's & Dementia: Diagnosis, Assessment & Disease Monitoring,* 9**,** 1-9.

LAO, P. J., HANDEN, B. L., BETTHAUSER, T. J., MIHAILA, I., HARTLEY, S. L., COHEN, A. D., TUDORASCU, D. L., BULOVA, P. D., LOPRESTI, B. J. & TUMULURU, R. V. 2018. Alzheimer-like pattern of hypometabolism emerges with elevated amyloid-β burden in Down syndrome. *Journal of Alzheimer's Disease,* 61**,** 631-644.

LAPASSET, L., MILHAVET, O., PRIEUR, A., BESNARD, E., BABLED, A., AÏT-HAMOU, N., LESCHIK, J., PELLESTOR, F., RAMIREZ, J.-M. & DE VOS, J. 2011. Rejuvenating senescent and centenarian human cells by reprogramming through the pluripotent state. *Genes & development,* 25**,** 2248-2253.

LAVAUTE, T. M., YOO, Y. D., PANKRATZ, M. T., WEICK, J. P., GERSTNER, J. R. & ZHANG, S. C. 2009. Regulation of neural specification from human embryonic stem cells by BMP and FGF. *Stem Cells,* 27**,** 1741-1749.

LEE, C. T., BENDRIEM, R. M., KINDBERG, A. A., WORDEN, L. T., WILLIAMS, M. P., DRGON, T., MALLON, B. S., HARVEY, B. K., RICHIE, C. T., HAMILTON, R. S., CHEN, J., ERRICO, S. L., TSAI, S. Y., UHL, G. R. & FREED, W. J. 2015. Functional

consequences of 17q21.31/WNT3-WNT9B amplification in hPSCs with respect to neural differentiation. *Cell Rep,* 10**,** 616-32.

LEJEUNE, J. T. R. G. M. 1959. Le mogolisme, premier exemple d'aberration autosomique humaine. *Ann Genet,* 1**,** 41-49.

LEUNG, A. W., MURDOCH, B., SALEM, A. F., PRASAD, M. S., GOMEZ, G. A. & GARCÍA-CASTRO, M. I. 2016. WNT/β-catenin signaling mediates human neural crest induction via a pre-neural border intermediate. *Development,* 143**,** 398-410.

LI, L. B., CHANG, K. H., WANG, P. R., HIRATA, R. K., PAPAYANNOPOULOU, T. & RUSSELL, D. W. 2012. Trisomy correction in down syndrome induced pluripotent stem cells. *Cell Stem Cell,* 11**,** 615-619.

LI, X. J., ZHANG, X., JOHNSON, M. A., WANG, Z. B., LAVAUTE, T. & ZHANG, S. C. 2009. Coordination of sonic hedgehog and Wnt signaling determines ventral and dorsal telencephalic neuron types from human embryonic stem cells. *Development,* 136**,** 4055-4063.

LI, Z., KLEIN, J. A., RAMPAM, S., KURZION, R., CAMPBELL, N. B., PATEL, Y., HAYDAR, T. F. & ZELDICH, E. 2022. Asynchronous excitatory neuron development in an isogenic cortical spheroid model of Down syndrome. *Front Neurosci,* 16**,** 932384.

LI, Z. Y., YU, T., MORISHIMA, M., PAO, A., LADUCA, J., CONROY, J., NOWAK, N., MATSUI, S. I., SHIRAISHI, I. & YU, Y. E. 2007. Duplication of the entire 22.9 Mb human chromosome 21 syntenic region on mouse chromosome 16 causes cardiovascular and gastrointestinal abnormalities. *Human Molecular Genetics,* 16**,** 1359-1366.

LIN, S.-C. & NICOLELIS, M. A. 2008. Neuronal ensemble bursting in the basal forebrain encodes salience irrespective of valence. *Neuron,* 59**,** 138-149.

LIU, J., XIAO, Q., XIAO, J., NIU, C., LI, Y., ZHANG, X., ZHOU, Z., SHU, G. & YIN, G. 2022. Wnt/β-catenin signalling: function, biological mechanisms, and therapeutic opportunities. *Signal Transduct Target Ther,* 7**,** 3.

LIU, P. P., XIE, Y., MENG, X. Y. & KANG, J. S. 2019. History and progress of hypotheses and clinical trials for Alzheimer's disease. *Signal Transduction and Targeted Therapy,* 4.

LIU, Y., LIU, H., SAUVEY, C., YAO, L., ZARNOWSKA, E. D. & ZHANG, S. C. 2013a. Directed differentiation of forebrain GABA interneurons from human pluripotent stem cells. *Nat Protoc,* 8**,** 1670-9.

LIU, Y., WEICK, J. P., LIU, H., KRENCIK, R., ZHANG, X., MA, L., ZHOU, G.-M., AYALA, M. & ZHANG, S.-C. 2013b. Medial ganglionic eminence–like cells derived from human embryonic stem cells correct learning and memory deficits. *Nature Biotechnology,* 31**,** 440-447.

LOCKROW, J., BOGER, H., BIMONTE-NELSON, H. & GRANHOLM, A. C. 2011. Effects of long-term memantine on memory and neuropathology in Ts65Dn mice, a model for Down syndrome. *Behavioural Brain Research,* 221**,** 610-622.

LOCKROW, J., PRAKASAM, A., HUANG, P., BIMONTE-NELSON, H., SAMBAMURTI, K. & GRANHOLM, A. C. 2009. Cholinergic degeneration and memory loss delayed by vitamin E in a Down syndrome mouse model. *Exp Neurol,* 216**,** 278-89.

LONG, K. R. & HUTTNER, W. B. 2019. How the extracellular matrix shapes neural development. *Open Biology,* 9**,** 180216.

LONG, K. R. & HUTTNER, W. B. 2021. The Role of the Extracellular Matrix in Neural Progenitor Cell Proliferation and Cortical Folding During Human Neocortex Development. *Front Cell Neurosci,* 15**,** 804649.

LOPEZ-COVIELLA, I., FOLLETTIE, M. T., MELLOTT, T. J., KOVACHEVA, V. P., SLACK, B. E., DIESL, V., BERSE, B., THIES, R. S. & BLUSZTAJN, J. K. 2005. Bone morphogenetic protein 9 induces the transcriptome of basal forebrain cholinergic neurons. *Proc Natl Acad Sci U S A,* 102**,** 6984-9.

LOTT, I. T. & HEAD, E. 2019. Dementia in Down syndrome: unique insights for Alzheimer disease research. *Nature Reviews Neurology,* 15**,** 135-147.

LOTT, I. T., HEAD, E., DORAN, E. & BUSCIGLIO, J. 2006. Beta-amyloid, oxidative stress and down syndrome. *Curr Alzheimer Res,* 3**,** 521-8.

LOVE, M. I., HUBER, W. & ANDERS, S. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology,* 15**,** 550.

LU, H. E., YANG, Y. C., CHEN, S. M., SU, H. L., HUANG, P. C., TSAI, M. S., WANG, T. H., TSENG, C. P. & HWANG, S. M. 2013. Modeling neurogenesis impairment in down syndrome with induced pluripotent stem cells from Trisomy 21 amniotic fluid cells. *Experimental Cell Research,* 319**,** 498-505.

LU, J., LIAN, G., ZHOU, H., ESPOSITO, G., STEARDO, L., DELLI-BOVI, L. C., HECHT, J. L., LU, Q. R. & SHEEN, V. 2012. OLIG2 over-expression impairs proliferation of human Down syndrome neural progenitors. *Hum Mol Genet,* 21**,** 2330-40.

MA, S., ZANG, T., LIU, M. L. & ZHANG, C. L. 2020. Aging-relevant human basal forebrain cholinergic neurons as a cell model for Alzheimer's disease. *Mol Neurodegener,* 15**,** 61.

MACLEAN, G. A., MENNE, T. F., GUO, G. J., SANCHEZ, D. J., PARK, I. H., DALEY, G. Q. & ORKIN, S. H. 2012. Altered hematopoiesis in trisomy 21 as revealed through in vitro differentiation of isogenic human pluripotent cells. *Proceedings of the National Academy of Sciences of the United States of America,* 109**,** 17567-17572.

MANABE, T., TATSUMI, K., INOUE, M., MAKINODAN, M., YAMAUCHI, T., MAKINODAN, E., YOKOYAMA, S., SAKUMURA, R. & WANAKA, A. 2007. L3/Lhx8 is a pivotal factor for cholinergic differentiation of murine embryonic stem cells. *Cell Death Differ,* 14**,** 1080-5.

MANDAS, A., MEREU, R. M., CATTE, O., SABA, A., SERCHISU, L., COSTAGGIU, D., PEIRETTI, E., CAMINITI, G., VINCI, M., CASU, M., PILUDU, S., FOSSARELLO, M., MANCONI, P. E. & DESSI, S. 2014. Cognitive impairment and age-related vision disorders: their possible relationship and the evaluation of the use of aspirin and statins in a 65 years-and-over Sardinian population. *Frontiers in Aging Neuroscience,* 6.

MARKS, N. & BERG, M. J. 2003. APP processing enzymes (secretases) as therapeutic targets: insights from the use of transgenics (Tgs) and transfected cells. *Neurochemical research,* 28**,** 1049-1062.

MAROOF, A. M., KEROS, S., TYSON, J. A., YING, S.-W., GANAT, Y. M., MERKLE, F. T., LIU, B., GOULBURN, A., STANLEY, E. G. & ELEFANTY, A. G. 2013. Directed differentiation and functional maturation of cortical interneurons from human embryonic stem cells. *Cell stem cell,* 12**,** 559-572.

MATSUMOTO, K., TANAKA, T., FURUYAMA, T., KASHIHARA, Y., MORI, T., ISHII, N., KITANAKA, J., TAKEMURA, M., TOHYAMA, M. & WANAKA, A. 1996. L3, a novel murine LIM-homeodomain transcription factor expressed in the ventral telencephalon and the mesenchyme surrounding the oral cavity. *Neurosci Lett,* 204**,** 113-6.

MATTSON, M. P. & MAGNUS, T. 2006. Ageing and neuronal vulnerability. *Nature Reviews Neuroscience,* 7**,** 278-294.

MCCANN, B., LEVMAN, J., BAUMER, N., LAM, M. Y., SHIOHAMA, T., COGGER, L., MACDONALD, A., IJNER, P. & TAKAHASHI, E. 2021. Structural magnetic resonance imaging demonstrates volumetric brain abnormalities in down syndrome: Newborns to young adults. *Neuroimage Clin,* 32**,** 102815.

MCNAUGHTON, D., KNIGHT, W., GUERREIRO, R., RYAN, N., LOWE, J., POULTER, M., NICHOLL, D. J., HARDY, J., REVESZ, T. & LOWE, J. 2012. Duplication of amyloid precursor protein (APP), but not prion protein (PRNP) gene is a significant cause of early onset dementia in a large UK series. *Neurobiology of aging,* 33**,** 426. e13-426. e21.

MEDA, L., CASSATELLA, M. A., SZENDREI, G. I., OTVOS, L., JR., BARON, P., VILLALBA, M., FERRARI, D. & ROSSI, F. 1995. Activation of microglial cells by beta-amyloid protein and interferon-gamma. *Nature,* 374**,** 647-50.

MEHARENA, H. S., MARCO, A., DILEEP, V., LOCKSHIN, E. R., AKATSU, G. Y., MULLAHOO, J., WATSON, L. A., KO, T., GUERIN, L. N., ABDURROB, F., RENGARAJAN, S., PAPANASTASIOU, M., JAFFE, J. D. & TSAI, L. H. 2022. Down-syndrome-induced senescence disrupts the nuclear architecture of neural progenitors. *Cell Stem Cell,* 29**,** 116-130 e7.

MENENDEZ, J. A., VELLON, L., OLIVERAS-FERRAROS, C., CUFÍ, S. & VAZQUEZ-MARTIN, A. 2011. mTOR-regulated senescence and autophagy during reprogramming of somatic cells to pluripotency: a roadmap from energy metabolism to stem cell renewal and aging. *Cell cycle,* 10**,** 3658-3677.

MENENDEZ, M. 2005. Down syndrome, Alzheimer's disease and seizures. *Brain Dev.,* 27**,** 246-252.

MENGEL, D., LIU, W., GLYNN, R. J., SELKOE, D. J., STRYDOM, A., LAI, F., ROSAS, H. D., TORRES, A., PATSIOGIANNIS, V., SKOTKO, B. & WALSH, D. M. 2020. Dynamics of plasma biomarkers in Down syndrome: the relative levels of A beta 42 decrease with age, whereas NT1 tau and NfL increase. *Alzheimers Research & Therapy,* 12.

MERTENS, J., PAQUOLA, A. C. M., KU, M., HATCH, E., BÖHNKE, L., LADJEVARDI, S., MCGRATH, S., CAMPBELL, B., LEE, H., HERDY, J. R., GONÇALVES, J. T., TODA, T., KIM, Y., WINKLER, J., YAO, J., HETZER, M. W. & GAGE, F. H. 2015. Directly Reprogrammed Human Neurons Retain Aging-Associated Transcriptomic Signatures and Reveal Age-Related Nucleocytoplasmic Defects. *Cell Stem Cell,* 17**,** 705-718.

MESULAM, M., SHAW, P., MASH, D. & WEINTRAUB, S. 2004. Cholinergic nucleus basalis tauopathy emerges early in the aging-MCI-AD continuum. *Annals of Neurology: Official Journal of the American Neurological Association and the Child Neurology Society,* 55**,** 815-828.

MESULAM, M. M., MUFSON, E. J., LEVEY, A. I. & WAINER, B. H. 1983. Cholinergic innervation of cortex by the basal forebrain: cytochemistry and cortical connections of the septal area, diagonal band nuclei, nucleus basalis (substantia innominata), and hypothalamus in the rhesus monkey. *J Comp Neurol,* 214**,** 170-97.

MEYNERT, T. 1872. Handbuch der Lehre von den Geweben des Menschen und der Thiere. *Stricker S, editor,* 1**,** 694-808.

MICHAUD, M., BALARDY, L., MOULIS, G., GAUDIN, C., PEYROT, C., VELLAS, B., CESARI, M. & NOURHASHEMI, F. 2013. Proinflammatory cytokines, aging, and age-related diseases. *J Am Med Dir Assoc,* 14**,** 877-82.

MIETTINEN, R. A., KALESNYKAS, G. & KOIVISTO, E. H. 2002. Estimation of the total number of cholinergic neurons containing estrogen receptor-alpha in the rat basal forebrain. *J Histochem Cytochem,* 50**,** 891-902.

MIKKELSEN, M. 1977. Down syndrome: cytogenetical epidemiology. *Hereditas,* 86**,** 45-50.

MILLER, J. D., GANAT, Y. M., KISHINEVSKY, S., BOWMAN, R. L., LIU, B., TU, E. Y., MANDAL, P. K., VERA, E., SHIM, J.-W. & KRIKS, S. 2013. Human iPSC-based modeling of late-onset disease via progerin-induced aging. *Cell stem cell,* 13**,** 691-705.

MILNE, M. R., HAUG, C. A., ÁBRAHÁM, I. M. & KWAKOWSKY, A. 2015. Estradiol Modulation of Neurotrophin Receptor Expression in Female Mouse Basal Forebrain Cholinergic Neurons In Vivo. *Endocrinology,* 156**,** 613-626.

MITSUSHIMA, D., SANO, A. & TAKAHASHI, T. 2013. A cholinergic trigger drives learning-induced plasticity at hippocampal synapses. *Nature communications,* 4**,** 1-10.

MOLLO, N., AURILIA, M., SCOGNAMIGLIO, R., ZERILLO, L., CICATIELLO, R., BONFIGLIO, F., PAGANO, P., PALADINO, S., CONTI, A., NITSCH, L. & IZZO, A. 2022. Overexpression of the Hsa21 Transcription Factor RUNX1 Modulates the Extracellular Matrix in Trisomy 21 Cells. *Frontiers in Genetics,* 13.

MORI, H. 1997. The biological significance of neuropathological lesions in Alzheimer's disease. *Neurobiol.Aging,* 18**,** 379-382.

MORI, T., YUXING, Z., TAKAKI, H., TAKEUCHI, M., ISEKI, K., HAGINO, S., KITANAKA, J., TAKEMURA, M., MISAWA, H., IKAWA, M., OKABE, M. & WANAKA, A. 2004. The LIM homeobox gene, L3/Lhx8, is necessary for proper development of basal forebrain cholinergic neurons. *Eur J Neurosci,* 19**,** 3129-41.

MOU, X. N., WU, Y. B., CAO, H. H., MENG, Q. Z., WANG, Q. H., SUN, C. C., HU, S. S., MA, Y. & ZHANG, H. 2012. Generation of disease-specific induced pluripotent stem cells from patients with different karyotypes of Down syndrome. *Stem Cell Research & Therapy,* 3.

MOUTON-LIGER, F., SAHÚN, I., COLLIN, T., LOPES PEREIRA, P., MASINI, D., THOMAS, S., PALY, E., LUILIER, S., MÊME, S., JOUHAULT, Q., BENNAÏ, S., BELOEIL, J. C., BIZOT, J. C., HÉRAULT, Y., DIERSSEN, M. & CRÉAU, N. 2014. Developmental molecular and functional cerebellar alterations induced by PCP4/PEP19 overexpression: implications for Down syndrome. *Neurobiol Dis,* 63**,** 92-106.

MOUTON-LIGER, F., THOMAS, S., RATTENBACH, R., MAGNOL, L., LARIGALDIE, V., LEDRU, A., HERAULT, Y., VERNEY, C. & CRÉAU, N. 2011. PCP4 (PEP19) overexpression induces premature neuronal differentiation associated with Ca(2+) /calmodulin-dependent kinase II-δ activation in mouse models of Down syndrome. *J Comp Neurol,* 519**,** 2779-802.

MUCHOVÁ, J., ŽITŇANOVÁ, I. & ĎURAČKOVÁ, Z. 2014. Oxidative stress and Down syndrome. Do antioxidants play a role in therapy? *Physiol Res,* 63**,** 535-42.

MUFSON, E., COUNTS, S., CHE, S. & GINSBERG, S. cDNA array and quantitative PCR analysis of neurotrophin receptor transcripts in cholinergic basal forebrain neurons in people with mild cognitive impairment (MCI) and Alzheimer's disease. Proc. Soc. Neurosci, 2004. 335-336.

MUFSON, E. J., BOTHWELL, M., HERSH, L. B. & KORDOWER, J. H. 1989a. Nerve growth factor receptor immunoreactive profiles in the normal, aged human basal forebrain: Colocalization with cholinergic neurons. *Journal of Comparative Neurology,* 285**,** 196-217.

MUFSON, E. J., BOTHWELL, M. & KORDOWER, J. H. 1989b. Loss of nerve growth factor receptor-containing neurons in Alzheimer's disease: a quantitative analysis across subregions of the basal forebrain. *Exp Neurol,* 105**,** 221-32.

MUFSON, E. J., CONNER, J. M. & KORDOWER, J. H. 1995. Nerve growth factor in Alzheimer's disease: defective retrograde transport to nucleus basalis. *Neuroreport,* 6**,** 1063-6.

MUFSON, E. J., GINSBERG, S. D., IKONOMOVIC, M. D. & DEKOSKY, S. T. 2003. Human cholinergic basal forebrain: chemoanatomy and neurologic dysfunction. *Journal of Chemical Neuroanatomy,* 26**,** 233-242.

MUFSON, E. J., LI, J. M., SOBREVIELA, T. & KORDOWER, J. H. 1996. Decreased trkA gene expression within basal forebrain neurons in Alzheimer's disease. 8**,** 29.

MUFSON, E. J., MA, S. Y., DILLS, J., COCHRAN, E. J., LEURGANS, S., WUU, J., BENNETT, D. A., JAFFAR, S., GILMOR, M. L., LEVEY, A. I. & KORDOWER, J. H. 2002. Loss of basal forebrain P75NTR immunoreactivity in subjects with mild cognitive impairment and Alzheimer's disease. *Journal of Comparative Neurology,* 443**,** 136-153.

MULLIGAN, K. A. & CHEYETTE, B. N. 2012. Wnt signaling in vertebrate neural development and function. *J Neuroimmune Pharmacol,* 7**,** 774-87.

MUÑOZ, S. S., ENGEL, M., BALEZ, R., DO-HA, D., CABRAL-DA-SILVA, M. C., HERNÁNDEZ, D., BERG, T., FIFITA, J. A., GRIMA, N., YANG, S., BLAIR, I. P., NICHOLSON, G., COOK, A. L., HEWITT, A. W., PÉBAY, A. & OOI, L. 2020. A Simple Differentiation Protocol for Generation of Induced Pluripotent Stem Cell-Derived Basal Forebrain-Like Cholinergic Neurons for Alzheimer's Disease and Frontotemporal Dementia Disease Modeling. *Cells,* 9.

MURRAY, A., LETOURNEAU, A., CANZONETTA, C., STATHAKI, E., GIMELLI, S., SLOAN-BENA, F., ABREHART, R., GOH, P., LIM, S., BALDO, C., DAGNA-BRICARELLI, F., HANNAN, S., MORTENSEN, M., BALLARD, D., COURT, D. S., FUSAKI, N., HASEGAWA, M., SMART, T. G., BISHOP, C., ANTONARAKIS, S. E., GROET, J. & NIZETIC, D. 2015a. Brief Report: Isogenic Induced Pluripotent Stem Cell Lines From an Adult With Mosaic Down Syndrome Model Accelerated Neuronal Ageing and Neurodegeneration. *Stem Cells,* 33**,** 2077-2084.

MURRAY, A., LETOURNEAU, A., CANZONETTA, C., STATHAKI, E., GIMELLI, S., SLOAN-BENA, F., ABREHART, R., GOH, P., LIM, S., BALDO, C., DAGNA-BRICARELLI, F., HANNAN, S., MORTENSEN, M., BALLARD, D., SYNDERCOMBE COURT, D., FUSAKI, N., HASEGAWA, M., SMART, T. G., BISHOP, C., ANTONARAKIS, S. E., GROET, J. & NIZETIC, D. 2015b. Brief report: isogenic induced pluripotent stem cell lines from an adult with mosaic down syndrome model accelerated neuronal ageing and neurodegeneration. *Stem Cells,* 33**,** 2077-84.

NARDONE, R., MARTH, R., AUSSERER, H., BRATTI, A. & TEZZON, F. 2006. Reduced short latency afferent inhibition in patients with Down syndrome and Alzheimer-type dementia. *Clinical Neurophysiology,* 117**,** 2204-2210.

NAUMANN, T., CASADEMUNT, E., HOLLERBACH, E., HOFMANN, J., DECHANT, G., FROTSCHER, M. & BARDE, Y.-A. 2002. Complete Deletion of the Neurotrophin Receptor p75NTRLeads to Long-Lasting Increases in the Number of Basal Forebrain Cholinergic Neurons. *Journal of Neuroscience,* 22**,** 2409-2418.

NEVES, G., SHAH, M. M., LIODIS, P., ACHIMASTOU, A., DENAXA, M., ROALFE, G., SESAY, A., WALKER, M. C. & PACHNIS, V. 2013. The LIM homeodomain protein

Lhx6 regulates maturation of interneurons and network excitability in the mammalian cortex. *Cereb Cortex,* 23**,** 1811-23.

NICHOLAS, C. R., CHEN, J., TANG, Y., SOUTHWELL, D. G., CHALMERS, N., VOGT, D., ARNOLD, C. M., CHEN, Y.-J. J., STANLEY, E. G. & ELEFANTY, A. G. 2013. Functional maturation of hPSC-derived forebrain interneurons requires an extended timeline and mimics human neural development. *Cell stem cell,* 12**,** 573-586.

NÓBREGA-PEREIRA, S., KESSARIS, N., DU, T., KIMURA, S., ANDERSON, S. A. & MARÍN, O. 2008. Postmitotic Nkx2-1 controls the migration of telencephalic interneurons by direct repression of guidance receptors. *Neuron,* 59**,** 733-45.

NUKINA, N. & IHARA, Y. 1986. ONE OF THE ANTIGENIC DETERMINANTS OF PAIRED HELICAL FILAMENTS IS RELATED TO TAU PROTEIN. *Journal of Biochemistry,* 99**,** 1541-1544.

OAKLEY, H., COLE, S. L., LOGAN, S., MAUS, E., SHAO, P., CRAFT, J., GUILLOZET-BONGAARTS, A., OHNO, M., DISTERHOFT, J., VAN ELDIK, L., BERRY, R. & VASSAR, R. 2006. Intraneuronal beta-amyloid aggregates, neurodegeneration, and neuron loss in transgenic mice with five familial Alzheimer's disease mutations: potential factors in amyloid plaque formation. *J Neurosci,* 26**,** 10129-40.

OLDAK, B., WILDSCHUTZ, E., BONDARENKO, V., COMAR, M.-Y., ZHAO, C., AGUILERA-CASTREJON, A., TARAZI, S., VIUKOV, S., PHAM, T. X. A., ASHOUOKHI, S., LOKSHTANOV, D., RONCATO, F., ARIEL, E., ROSE, M., LIVNAT, N., SHANI, T., JOUBRAN, C., COHEN, R., ADDADI, Y., CHEMLA, M., KEDMI, M., KEREN-SHAUL, H., PASQUE, V., PETROPOULOS, S., LANNER, F., NOVERSHTERN, N. & HANNA, J. H. 2023. Complete human day 14 post-implantation embryo models from naive ES cells. *Nature,* 622**,** 562-573.

OLMOS-SERRANO, J. L., KANG, H. J., TYLER, W. A., SILBEREIS, J. C., CHENG, F., ZHU, Y., PLETIKOS, M., JANKOVIC-RAPAN, L., CRAMER, N. P., GALDZICKI, Z., GOODLIFFE, J., PETERS, A., SETHARES, C., DELALLE, I., GOLDEN, J. A., HAYDAR, T. F. & SESTAN, N. 2016. Down Syndrome Developmental Brain Transcriptome Reveals Defective Oligodendrocyte Differentiation and Myelination. *Neuron,* 89**,** 1208-1222.

OVCHINNIKOV, D. A., KORN, O., VIRSHUP, I., WELLS, C. A. & WOLVETANG, E. J. 2018. The Impact of APP on Alzheimer-like Pathogenesis and Gene Expression in Down Syndrome iPSC-Derived Neurons. *Stem Cell Reports,* 11**,** 32-42.

PANKRATZ, M. T., LI, X. J., LAVAUTE, T. M., LYONS, E. A., CHEN, X. & ZHANG, S. C. 2007. Directed Neural Differentiation of hESCs via an Obligated Primitive Anterior Stage. *Stem Cells,* 25**,** 1511-1520.

PAPAVASSILIOU, P., YORK, T. P., GURSOY, N., HILL, G., NICELY, L. V., SUNDARAM, U., MCCLAIN, A., AGGEN, S. H., EAVES, L., RILEY, B. & JACKSON-COOK, C. 2009. The phenotype of persons having mosaicism for trisomy 21/Down syndrome reflects the percentage of trisomic cells present in different tissues. *Am J Med Genet A,* 149a**,** 573-83.

PARIKH, V. & SARTER, M. 2008. Cholinergic mediation of attention.

PARK, I. H., ARORA, N., HUO, H., MAHERALI, N., AHFELDT, T., SHIMAMURA, A., LENSCH, M. W., COWAN, C., HOCHEDLINGER, K. & DALEY, G. Q. 2008a. Disease-specific induced pluripotent stem cells. *Cell,* 134**,** 877-886.

PARK, I. H., ZHAO, R., WEST, J. A., YABUUCHI, A., HUO, H., INCE, T. A., LEROU, P. H., LENSCH, M. W. & DALEY, G. Q. 2008b. Reprogramming of human somatic cells to pluripotency with defined factors. *Nature,* 451**,** 141-146.

PASQUINI, G., ROJO ARIAS, J. E., SCHÄFER, P. & BUSSKAMP, V. 2021. Automated methods for cell type annotation on scRNA-seq data. *Computational and Structural Biotechnology Journal,* 19**,** 961-969.

PATAPOUTIAN, A. & REICHARDT, L. F. 2000. Roles of Wnt proteins in neural development and maintenance. *Curr Opin Neurobiol,* 10**,** 392-9.

PATKEE, P. A., BABURAMANI, A. A., KYRIAKOPOULOU, V., DAVIDSON, A., AVINI, E., DIMITROVA, R., ALLSOP, J., HUGHES, E., KANGAS, J., MCALONAN, G. & RUTHERFORD, M. A. 2020. Early alterations in cortical and cerebellar regional brain growth in Down Syndrome: An in vivo fetal and neonatal MRI assessment. *Neuroimage Clin,* 25**,** 102139.

PEDROZA, M., GASSALOGLU, S. I., DIAS, N., ZHONG, L., HOU, T.-C. J., KRETZMER, H., SMITH, Z. D. & SOZEN, B. 2023. Self-patterning of human stem cells into post-implantation lineages. *Nature,* 622**,** 574-583.

PENNEY, J., RALVENIUS, W. T. & TSAI, L.-H. 2020. Modeling Alzheimer's disease with iPSC-derived brain cells. *Molecular Psychiatry,* 25**,** 148-167.

PEREZ, S. E., MIGUEL, J. C., HE, B., MALEK-AHMADI, M., ABRAHAMSON, E. E., IKONOMOVIC, M. D., LOTT, I., DORAN, E., ALLDRED, M. J. & GINSBERG, S. D. 2019. Frontal cortex and striatal cellular and molecular pathobiology in individuals with Down syndrome with and without dementia. *Acta neuropathologica,* 137**,** 413-436.

PERLUIGI, M. & BUTTERFIELD, D. A. 2012. Oxidative Stress and Down Syndrome: A Route toward Alzheimer-Like Dementia. *Curr.Gerontol.Geriatr.Res.,* 2012**,** 724904.

PERRY, E. K., JOHNSON, M., KERWIN, J. M., PIGGOTT, M. A., COURT, J. A., SHAW, P. J., INCE, P. G., BROWN, A. & PERRY, R. H. 1992. CONVERGENT CHOLINERGIC ACTIVITIES IN AGING AND ALZHEIMERS-DISEASE. 13**,** 400.

PIPINO, C., MUKHERJEE, S., DAVID, A. L., BLUNDELL, M. P., SHAW, S. W., SUNG, P., SHANGARIS, P., WATERS, J. J., ELLERSHAW, D., CAVAZZANA, M., MOSTOSLAVSKY, G., PANDOLFI, A., PIERRO, A., GUILLOT, P. V., THRASHER, A. J. & DE COPPI, P. 2014. Trisomy 21 Mid-Trimester Amniotic Fluid Induced Pluripotent Stem Cells Maintain Genetic Signatures During Reprogramming: Implications for Disease Modeling and Cryobanking. *Cellular Reprogramming,* 16**,** 331-344.

PITCHERS, K. K., PHILLIPS, K. B., JONES, J. L., ROBINSON, T. E. & SARTER, M. 2017. Diverse Roads to Relapse: A Discriminative Cue Signaling Cocaine Availability Is More Effective in Renewing Cocaine Seeking in Goal Trackers Than Sign Trackers and Depends on Basal Forebrain Cholinergic Activity. *J Neurosci,* 37**,** 7198-7208.

POWERS, B. E., KELLEY, C. M., VELAZQUEZ, R., ASH, J. A., STRAWDERMAN, M. S., ALLDRED, M. J., GINSBERG, S. D., MUFSON, E. J. & STRUPP, B. J. 2017. Maternal choline supplementation in a mouse model of Down syndrome: Effects on attention and nucleus basalis/substantia innominata neuron morphology in adult offspring. *Neuroscience,* 340**,** 501-514.

POWERS, B. E., VELAZQUEZ, R., KELLEY, C. M., ASH, J. A., STRAWDERMAN, M. S., ALLDRED, M. J., GINSBERG, S. D., MUFSON, E. J. & STRUPP, B. J. 2016.

Attentional function and basal forebrain cholinergic neuron morphology during aging in the Ts65Dn mouse model of Down syndrome. *Brain Struct Funct,* 221**,** 4337-4352.

PRASHER, V. P., FARRER, M. J., KESSLING, A. M., FISHER, E. M. C., WEST, R. J., BARBER, P. C. & BUTLER, A. C. 1998. Molecular mapping of Alzheimer-type dementia in Down's syndrome. *Annals of Neurology,* 43**,** 380-383.

PRESSON, A. P., PARTYKA, G., JENSEN, K. M., DEVINE, O. J., RASMUSSEN, S. A., MCCABE, L. L. & MCCABE, E. R. 2013. Current estimate of down syndrome population prevalence in the United States. *J Pediatr,* 163**,** 1163-8.

PRICE, D., KITT, C. A., HEDREEN, J., WHITEHOUSE, P., STRUBLE, R., CORK, L., WALKER, L., MOBLEY, W., CRAWFORD, G. & SALVATERRA, P. 1986. Basal forebrain cholinergic systems in primate brain: Anatomical organization and role in the pathology of aging and dementia. *Dynamics of Cholinergic Function***,** 235-242.

PRUTTON, K. M., MARENTETTE, J. O., MACLEAN, K. N. & ROEDE, J. R. 2023. Characterization of mitochondrial and metabolic alterations induced by trisomy 21 during neural differentiation. *Free Radical Biology and Medicine,* 196**,** 11-21.

QIU, J. J., LIU, Y. N., WEI, H., ZENG, F. & YAN, J. B. 2023. Single-cell RNA sequencing of neural stem cells derived from human trisomic iPSCs reveals the abnormalities during neural differentiation of Down syndrome. *Front Mol Neurosci,* 16**,** 1137123.

RAFII, M. S., ANCES, B. M., SCHUPF, N., KRINSKY-MCHALE, S. J., MAPSTONE, M., SILVERMAN, W., LOTT, I., KLUNK, W., HEAD, E. & CHRISTIAN, B. 2020. The AT (N) framework for Alzheimer's disease in adults with Down syndrome. *Alzheimer's & Dementia: Diagnosis, Assessment & Disease Monitoring,* 12**,** e12062.

RAFII, M. S., LUKIC, A. S., ANDREWS, R. D., BREWER, J., RISSMAN, R. A., STROTHER, S. C., WERNICK, M. N., PENNINGTON, C., MOBLEY, W. C. & NESS, S. 2017. PET imaging of tau pathology and relationship to amyloid, longitudinal MRI, and cognitive change in Down syndrome: results from the Down Syndrome Biomarker Initiative (DSBI). *Journal of Alzheimer's Disease,* 60**,** 439-450.

RANDO, T. A. & CHANG, H. Y. 2012. Aging, rejuvenation, and epigenetic reprogramming: resetting the aging clock. *Cell,* 148**,** 46-57.

REAL, R., PETER, M., TRABALZA, A., KHAN, S., SMITH, M. A., DOPP, J., BARNES, S. J., MOMOH, A., STRANO, A., VOLPI, E., KNOTT, G., LIVESEY, F. J. & DE PAOLA, V. 2018. In vivo modeling of human neuron dynamics and Down syndrome. *Science,* 362**,** 793-+.

REEVES, R. H., IRVING, N. G., MORAN, T. H., WOHN, A., KITT, C., SISODIA, S. S., SCHMIDT, C., BRONSON, R. T. & DAVISSON, M. T. 1995. A mouse model for Down syndrome exhibits learning and behaviour deficits. *Nat Genet,* 11**,** 177-84.

REMES, A. M., LARU, L., TUOMINEN, H., AALTO, S., KEMPPAINEN, N., MONONEN, H., NÅGREN, K., PARKKOLA, R. & RINNE, J. O. 2008. Carbon 11–labeled Pittsburgh Compound B positron emission tomographic amyloid imaging in patients with APP locus duplication. *Archives of neurology,* 65**,** 540-544.

RICHARDS, B. W. 1969. Mosaic mongolism. *J Ment Defic Res,* 13**,** 66-83.

RICHTER, N., DAVID, L. S., GROTHE, M. J., TEIPEL, S., DIETLEIN, M., TITTGEMEYER, M., NEUMAIER, B., FINK, G. R., ONUR, O. A. & KUKOLJA, J. 2022. Age and Anterior Basal Forebrain Volume Predict the Cholinergic Deficit in Patients with Mild Cognitive Impairment due to Alzheimer's Disease. *J Alzheimers Dis,* 86**,** 425-440.

ROIZEN, N. J. & PATTERSON, D. 2003. Down's syndrome. *Lancet,* 361**,** 1281-9.

RONG, S., LI, Y., LI, B., NIE, K., ZHANG, P., CAI, T., MEI, M., WANG, L. & ZHANG, Y. 2021. Meynert nucleus-related cortical thinning in Parkinson's disease with mild cognitive impairment. *Quant Imaging Med Surg,* 11**,** 1554-1566.

ROSS, M. H., GALABURDA, A. M. & KEMPER, T. L. 1984. Down's syndrome: is there a decreased population of neurons? *Neurology,* 34**,** 909-916.

ROTH, K. A. 2001. Caspases, Apoptosis, and Alzheimer Disease: Causation, Correlation, and Confusion. *Journal of Neuropathology & Experimental Neurology,* 60**,** 829-838.

ROVELET-LECRUX, A., FREBOURG, T., TUOMINEN, H., MAJAMAA, K., CAMPION, D. & REMES, A. M. 2007. APP locus duplication in a Finnish family with dementia and intracerebral haemorrhage. *Journal of Neurology, Neurosurgery & Psychiatry,* 78**,** 1158-1159.

ROVELET-LECRUX, A., HANNEQUIN, D., RAUX, G., LE MEUR, N., LAQUERRIÈRE, A., VITAL, A., DUMANCHIN, C., FEUILLETTE, S., BRICE, A. & VERCELLETTO, M. 2006. APP locus duplication causes autosomal dominant early-onset Alzheimer disease with cerebral amyloid angiopathy. *Nature genetics,* 38**,** 24-26.

ROZALEM ARANHA, M., IULITA, M. F., MONTAL, V., PEGUEROLES, J., BEJANIN, A., VAQUÉ-ALCÁZAR, L., GROTHE, M. J., CARMONA-IRAGUI, M., VIDELA, L., BENEJAM, B., ARRANZ, J., PADILLA, C., VALLDENEU, S., BARROETA, I., ALTUNA, M., FERNÁNDEZ, S., RIBAS, L., VALLE-TAMAYO, N., ALCOLEA, D., GONZÁLEZ-ORTIZ, S., BARGALLÓ, N., ZETTERBERG, H., BLENNOW, K., BLESA, R., WISNIEWSKI, T., BUSCIGLIO, J., CUELLO, A. C., LLEÓ, A. & FORTEA, J. 2023. Basal forebrain atrophy along the Alzheimer's disease continuum in adults with Down syndrome. *Alzheimer's & Dementia,* 19**,** 4817-4827.

RUSTED, J. 1994. Cholinergic blockade and human information processing: are we asking the right questions? *J Psychopharmacol,* 8**,** 54-9.

SAGO, H., CARLSON, E. J., SMITH, D. J., KILBRIDGE, J., RUBIN, E. M., MOBLEY, W. C., EPSTEIN, C. J. & HUANG, T. T. 1998. Ts1Cje, a partial trisomy 16 mouse model for Down syndrome, exhibits learning and behavioral abnormalities. *Proc.Natl.Acad.Sci.U.S.A,* 95**,** 6256-6261.

SAKURAI, T., NAGATA, R., YAMANAKA, A., KAWAMURA, H., TSUJINO, N., MURAKI, Y., KAGEYAMA, H., KUNITA, S., TAKAHASHI, S., GOTO, K., KOYAMA, Y., SHIODA, S. & YANAGISAWA, M. 2005. Input of orexin/hypocretin by a genetically encoded neurons revealed tracer in mice. *Neuron,* 46**,** 297-308.

SALEHI, A., DELCROIX, J. D., BELICHENKO, P. V., ZHAN, K., WU, C., VALLETTA, J. S., TAKIMOTO-KIMURA, R., KLESCHEVNIKOV, A. M., SAMBAMURTI, K., CHUNG, P. P., XIA, W., VILLAR, A., CAMPBELL, W. A., KULNANE, L. S., NIXON, R. A., LAMB, B. T., EPSTEIN, C. J., STOKIN, G. B., GOLDSTEIN, L. S. & MOBLEY, W. C. 2006. Increased App expression in a mouse model of Down's syndrome disrupts NGF transport and causes cholinergic neuron degeneration. *Neuron,* 51**,** 29-42.

SALEHI, A., DELCROIX, J. D. & SWAAB, D. F. 2004. Alzheimer's disease and NGF signaling. *Journal of Neural Transmission,* 111**,** 323-345.

SALEHI, A., VERHAAGEN, J., DIJKHUIZEN, P. A. & SWAAB, D. F. 1996. Co-localization of high-affinity neurotrophin receptors in nucleus basalis of Meynert neurons and their differential reduction in Alzheimer's disease. *Neuroscience,* 75**,** 373-387.

SANCHEZ, T., WANG, T., PEDRO, M. V., ZHANG, M., ESENCAN, E., SAKKAS, D., NEEDLEMAN, D. & SELI, E. 2018. Metabolic imaging with the use of fluorescence

lifetime imaging microscopy (FLIM) accurately detects mitochondrial dysfunction in mouse oocytes. *Fertility and Sterility,* 110**,** 1387-1397.

SANDERS, N. C., WILLIAMS, D. K. & WENGER, G. R. 2009. Does the learning deficit observed under an incremental repeated acquisition schedule of reinforcement in Ts65Dn mice, a model for Down syndrome, change as they age? *Behav Brain Res,* 203**,** 137-42.

SARTER, M., BRUNO, J. P. & GIVENS, B. 2003. Attentional functions of cortical cholinergic inputs: what does it mean for learning and memory? *Neurobiol Learn Mem,* 80**,** 245-56.

SAWADA, M., SAWADA, H. & NAGATSU, T. 2008. Effects of aging on neuroprotective and neurotoxic properties of microglia in neurodegenerative diseases. *Neurodegenerative Diseases,* 5**,** 254-256.

SAXENA, S. & CARONI, P. 2011. Selective neuronal vulnerability in neurodegenerative diseases: from stressor thresholds to degeneration. *Neuron,* 71**,** 35-48.

SCHEUNER, D., ECKMAN, C., JENSEN, M., SONG, X., CITRON, M., SUZUKI, N., BIRD, T., HARDY, J., HUTTON, M. & KUKULL, W. 1996. Secreted amyloid β–protein similar to that in the senile plaques of Alzheimer's disease is increased in vivo by the presenilin 1 and 2 and APP mutations linked to familial Alzheimer's disease. *Nature medicine,* 2**,** 864-870.

SCHMIDT-SIDOR, B., WISNIEWSKI, K. E., SHEPARD, T. H. & SERSEN, E. A. 1990. Brain growth in Down syndrome subjects 15 to 22 weeks of gestational age and birth to 60 months. *Clin.Neuropathol.,* 9**,** 181-190.

SCHMITZ, M. T., SANDOVAL, K., CHEN, C. P., MOSTAJO-RADJI, M. A., SEELEY, W. W., NOWAKOWSKI, T. J., YE, C. J., PAREDES, M. F. & POLLEN, A. A. 2022. The development and evolution of inhibitory neurons in primate cerebrum. *Nature,* 603**,** 871-877.

SCHMITZ, T. W. & DUNCAN, J. 2018. Normalization and the cholinergic microcircuit: a unified basis for attention. *Trends in Cognitive Sciences,* 22**,** 422-437.

SCHMITZ, T. W., SOREQ, H., POIRIER, J. & SPRENG, R. N. 2020. Longitudinal Basal Forebrain Degeneration Interacts with TREM2/C3 Biomarkers of Inflammation in Presymptomatic Alzheimer's Disease. *Journal of Neuroscience,* 40**,** 1931-1942.

SCHNITZLER, A. C., LOPEZ-COVIELLA, I. & BLUSZTAJN, J. K. 2008. Differential modulation of nerve growth factor receptor (p75) and cholinergic gene expression in purified p75-expressing and non-expressing basal forebrain neurons by BMP9. *Brain Res,* 1246**,** 19-28.

SCOTT, B. S., BECKER, L. E. & PETIT, T. L. 1983. Neurobiology of Down's syndrome. *Prog.Neurobiol.,* 21**,** 199-237.

SELKOE, D. J. 1991. THE MOLECULAR PATHOLOGY OF ALZHEIMERS-DISEASE. *Neuron,* 6**,** 487-498.

SELKOE, D. J. 2002. Alzheimer's disease is a synaptic failure. *Science,* 298**,** 789-91.

SELKOE, D. J. & HARDY, J. 2016. The amyloid hypothesis of Alzheimer's disease at 25years. *Embo Molecular Medicine,* 8**,** 595-608.

SEPULCRE, J., GROTHE, M. J., UQUILLAS, F. D. O., ORTIZ-TERÁN, L., DIEZ, I., YANG, H.-S., JACOBS, H. I., HANSEEUW, B. J., LI, Q. & EL-FAKHRI, G. 2018. Neurogenetic contributions to amyloid beta and tau spreading in the human cortex. *Nature medicine,* 24**,** 1910-1918.

SHARMA, V., NEHRA, S., DO, L. H., GHOSH, A., DESHPANDE, A. J. & SINGHAL, N. 2022. Biphasic cell cycle defect causes impaired neurogenesis in down syndrome. *Front Genet,* 13**,** 1007519.

SHEEDY, F. J., GREBE, A., RAYNER, K. J., KALANTARI, P., RAMKHELAWON, B., CARPENTER, S. B., BECKER, C. E., EDIRIWEERA, H. N., MULLICK, A. E., GOLENBOCK, D. T., STUART, L. M., LATZ, E., FITZGERALD, K. A. & MOORE, K. J. 2013. CD36 coordinates NLRP3 inflammasome activation by facilitating intracellular nucleation of soluble ligands into particulate ligands in sterile inflammation. *Nat Immunol,* 14**,** 812-20.

SHI, Y., INOUE, H., WU, J. C. & YAMANAKA, S. 2017. Induced pluripotent stem cell technology: a decade of progress. *Nat Rev Drug Discov,* 16**,** 115-130.

SHI, Y., KIRWAN, P., SMITH, J., ROBINSON, H. P. & LIVESEY, F. J. 2012. Human cerebral cortex development from pluripotent stem cells to functional excitatory synapses. *Nat.Neurosci.,* 15**,** 477-86, S1.

SHIN, M., BESSER, L. M., KUCIK, J. E., LU, C., SIFFEL, C. & CORREA, A. 2009. Prevalence of Down syndrome among children and adolescents in 10 regions of the United States. *Pediatrics,* 124**,** 1565-71.

SHUKKUR, E. A., SHIMOHATA, A., AKAGI, T., YU, W., YAMAGUCHI, M., MURAYAMA, M., CHUI, D., TAKEUCHI, T., AMANO, K., SUBRAMHANYA, K. H., HASHIKAWA, T., SAGO, H., EPSTEIN, C. J., TAKASHIMA, A. & YAMAKAWA, K. 2006. Mitochondrial dysfunction and tau hyperphosphorylation in Ts1Cje, a mouse model for Down syndrome. *Human Molecular Genetics,* 15**,** 2752-2762.

SINAI, A., MOKRYSZ, C., BERNAL, J., BOHNEN, I., BONELL, S., COURTENAY, K., DODD, K., GAZIZOVA, D., HASSIOTIS, A., HILLIER, R., MCBRIEN, J., MCCARTHY, J., MUKHERJI, K., NAEEM, A., PEREZ-ACHIAGA, N., RANTELL, K., SHARMA, V., THOMAS, D., WALKER, Z., WHITHAM, S. & STRYDOM, A. 2018. Predictors of Age of Diagnosis and Survival of Alzheimer's Disease in Down Syndrome. *J Alzheimers Dis,* 61**,** 717-728.

SLEEGERS, K., BROUWERS, N., GIJSELINCK, I., THEUNS, J., GOOSSENS, D., WAUTERS, J., DEL-FAVERO, J., CRUTS, M., DUIJN, C. M. V. & BROECKHOVEN, C. V. 2006. APP duplication is sufficient to cause early onset Alzheimer's dementia with cerebral amyloid angiopathy. *Brain,* 129**,** 2977-2983.

SLEIGH, J. N., BARREIRO-IGLESIAS, A., OLIVER, P. L., BIBA, A., BECKER, T., DAVIES, K. E., BECKER, C. G. & TALBOT, K. 2014. Chondrolectin affects cell survival and neuronal outgrowth in in vitro and in vivo models of spinal muscular atrophy. *Hum Mol Genet,* 23**,** 855-69.

SMITH, D. S. 2001. Health care management of adults with Down syndrome. *Am Fam Physician,* 64**,** 1031-8.

SNYDER, H. M., BAIN, L. J., BRICKMAN, A. M., CARRILLO, M. C., ESBENSEN, A. J., ESPINOSA, J. M., FERNANDEZ, F., FORTEA, J., HARTLEY, S. L., HEAD, E., HENDRIX, J., KISHNANI, P. S., LAI, F., LAO, P., LEMERE, C., MOBLEY, W., MUFSON, E. J., POTTER, H., ZAMAN, S. H., GRANHOLM, A. C., ROSAS, H. D., STRYDOM, A., WHITTEN, M. S. & RAFII, M. S. 2020. Further understanding the connection between Alzheimer's disease and Down syndrome. *Alzheimers Dement,* 16**,** 1065-1077.

SOBOL, M., KLAR, J., LAAN, L., SHAHSAVANI, M., SCHUSTER, J., ANNEREN, G., KONZER, A., MI, J., BERGQUIST, J., NORDLUND, J., HOEBER, J., HUSS, M., FALK, A. & DAHL, N. 2019. Transcriptome and Proteome Profiling of Neural Induced Pluripotent Stem Cells from Individuals with Down Syndrome Disclose Dynamic Dysregulations of Key Pathways and Cellular Functions. *Molecular Neurobiology,* 56**,** 7113-7127.

SOLARI, N. & HANGYA, B. 2018. Cholinergic modulation of spatial learning, memory and navigation. *European Journal of Neuroscience,* 48**,** 2199-2230.

STAGNI, F., GIACOMINI, A., EMILI, M., UGUAGLIATI, B., BONASONI, M. P., BARTESAGHI, R. & GUIDI, S. 2019. Subicular hypotrophy in fetuses with Down syndrome and in the Ts65Dn model of Down syndrome. *Brain Pathol,* 29**,** 366-379.

STARTIN, C. M., ASHTON, N. J., HAMBURG, S., HITHERSAY, R., WISEMAN, F. K., MOK, K. Y., HARDY, J., LLEO, A., LOVESTONE, S., PARNETTI, L., ZETTERBERG, H., HYE, A., FISHER, E., NIZETIC, D., HARDY, J., WESTON, R. L., TYBULEWICZ, V., KARMILOFF-SMITH, A., STRYDOM, A. & LONDOWN, S. C. 2019. Plasma biomarkers for amyloid, tau, and cytokines in Down syndrome and sporadic Alzheimer's disease. *Alzheimers Research & Therapy,* 11.

STEYER, B., BU, Q., CORY, E., JIANG, K., DUONG, S., SINHA, D., STELTZER, S., GAMM, D., CHANG, Q. & SAHA, K. 2018. Scarless Genome Editing of Human Pluripotent Stem Cells via Transient Puromycin Selection. *Stem Cell Reports,* 10**,** 642-654.

STRYDOM, A., COPPUS, A., BLESA, R., DANEK, A., FORTEA, J., HARDY, J., LEVIN, J., NUEBLING, G., REBILLAT, A.-S. & RITCHIE, C. 2018. Alzheimer's disease in Down syndrome: An overlooked population for prevention trials. *Alzheimer's & Dementia: Translational Research & Clinical Interventions,* 4**,** 703-713.

STURGEON, X. & GARDINER, K. J. 2011. Transcript catalogs of human chromosome 21 and orthologous chimpanzee and mouse regions. *Mamm Genome,* 22**,** 261-71.

SUN, J., CARLSON-STEVERMER, J., DAS, U., SHEN, M., DELENCLOS, M., SNEAD, A. M., WANG, L., LOI, J., PETERSEN, A. J., STOCKTON, M., BHATTACHARYYA, A., JONES, M. V., SPROUL, A. A., MCLEAN, P. J., ZHAO, X., SAHA, K. & ROY, S. 2018. A CRISPR/Cas9 based strategy to manipulate the Alzheimer's amyloid pathway. *bioRxiv***,** 310193.

SUN, X., KATO, H., SATO, H., HAN, X., HIROFUJI, Y., KATO, T. A., SAKAI, Y., OHGA, S., FUKUMOTO, S. & MASUDA, K. 2022. Dopamine-related oxidative stress and mitochondrial dysfunction in dopaminergic neurons differentiated from deciduous teeth-derived stem cells of children with Down syndrome. *FASEB Bioadv,* 4**,** 454-467.

SUSSEL, L., MARIN, O., KIMURA, S. & RUBENSTEIN, J. L. 1999. Loss of Nkx2.1 homeobox gene function results in a ventral to dorsal molecular respecification within the basal telencephalon: evidence for a transformation of the pallidum into the striatum. *Development,* 126**,** 3359-70.

SWAMINATHAN, S., HUENTELMAN, M. J., CORNEVEAUX, J. J., MYERS, A. J., FABER, K. M., FOROUD, T., MAYEUX, R., SHEN, L., KIM, S. & TURK, M. 2012. Analysis of copy number variation in Alzheimer's disease in a cohort of clinically characterized and neuropathologically verified individuals. *PloS one,* 7**,** e50640.

SWATTON, J. E., SELLERS, L. A., FAULL, R. L., HOLLAND, A., IRITANI, S. & BAHN, S. 2004. Increased MAP kinase activity in Alzheimer's and Down syndrome but not in schizophrenia human brain. *Eur J Neurosci,* 19**,** 2711-9.

SWEENEY, J. E., HOHMANN, C. F., OSTER-GRANITE, M. L. & COYLE, J. T. 1989. Neurogenesis of the basal forebrain in euploid and trisomy 16 mice: an animal model for developmental disorders in Down syndrome. *Neuroscience,* 31**,** 413-25.

TAKAHASHI, K., TANABE, K., OHNUKI, M., NARITA, M., ICHISAKA, T., TOMODA, K. & YAMANAKA, S. 2007. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell,* 131**,** 861-872.

TAO, Y. & ZHANG, S. C. 2016. Neural Subtype Specification from Human Pluripotent Stem Cells. *Cell Stem Cell,* 19**,** 573-586.

TARUI, T., IM, K., MADAN, N., MADANKUMAR, R., SKOTKO, B. G., SCHWARTZ, A., SHARR, C., RALSTON, S. J., KITANO, R., AKIYAMA, S., YUN, H. J., GRANT, E. & BIANCHI, D. W. 2020. Quantitative MRI Analyses of Regional Brain Growth in Living Fetuses with Down Syndrome. *Cereb Cortex,* 30**,** 382-390.

TAYEBATI, S. K., CECCHI, A., MARTINELLI, I., CARBONI, E. & AMENTA, F. 2019. Pharmacotherapy of Down's Syndrome: When and Which? *Cns & Neurological Disorders-Drug Targets,* 18**,** 750-757.

TELIAS, M. & BEN-YOSEF, D. 2021. Pharmacological Manipulation of Wnt/β-Catenin Signaling Pathway in Human Neural Precursor Cells Alters Their Differentiation Potential and Neuronal Yield. *Front Mol Neurosci,* 14**,** 680018.

TERRY, R. D., MASLIAH, E., SALMON, D. P., BUTTERS, N., DETERESA, R., HILL, R., HANSEN, L. A. & KATZMAN, R. 1991. Physical basis of cognitive alterations in Alzheimer's disease: synapse loss is the major correlate of cognitive impairment. *Ann Neurol,* 30**,** 572-80.

THAL, D. R., RÜB, U., ORANTES, M. & BRAAK, H. 2002. Phases of Aβ-deposition in the human brain and its relevance for the development of AD. *Neurology,* 58**,** 1791-1800.

THOMSON, J. A., ITSKOVITZ-ELDOR, J., SHAPIRO, S. S., WAKNITZ, M. A., SWIERGIEL, J. J., MARSHALL, V. S. & JONES, J. M. 1998. Embryonic stem cell lines derived from human blastocysts. *Science,* 282**,** 1145-1147.

THONBERG, H., FALLSTRÖM, M., BJÖRKSTRÖM, J., SCHOUMANS, J., NENNESMO, I. & GRAFF, C. 2011. Mutation screening of patients with Alzheimer disease identifies APP locus duplication in a Swedish patient. *BMC research notes,* 4**,** 1-9.

TIBERI, L., VANDERHAEGHEN, P. & VAN DEN AMEELE, J. 2012. Cortical neurogenesis and morphogens: diversity of cues, sources and functions. *Curr Opin Cell Biol,* 24**,** 269-76.

TOKUDA, T., FUKUSHIMA, T., IKEDA, S. I., SEKIJIMA, Y., SHOJI, S. I., YANAGISAWA, N. & TAMAOKA, A. 1997. Plasma levels of amyloid β proteins Aβ1–40 and Aβ1–42 (43) are elevated in Down's syndrome. *Annals of Neurology: Official Journal of the American Neurological Association and the Child Neurology Society,* 41**,** 271-273.

TOMIOKA, T., SHIMAZAKI, T., YAMAUCHI, T., OKI, T., OHGOH, M. & OKANO, H. 2014. LIM homeobox 8 (Lhx8) is a key regulator of the cholinergic neuronal function via a tropomyosin receptor kinase A (TrkA)-mediated positive feedback loop. *J Biol Chem,* 289**,** 1000-10.

TRAMUTOLA, A., LANZILLOTTA, C., BARONE, E., ARENA, A., ZULIANI, I., MOSCA, L., BLARZINO, C., BUTTERFIELD, D. A., PERLUIGI, M. & DI DOMENICO, F.

2018. Intranasal rapamycin ameliorates Alzheimer-like cognitive decline in a mouse model of Down syndrome. *Translational Neurodegeneration,* 7.

TRAXLER, L., LAGERWALL, J., EICHHORNER, S., STEFANONI, D., D'ALESSANDRO, A. & MERTENS, J. 2021. Metabolism navigates neural cell fate in development, aging and neurodegeneration. *Dis Model Mech,* 14.

TUDORASCU, D., LAYMON, C., ZAMMIT, M., MINHAS, D., ANDERSON, S., ELLISON, P., ZAMAN, S., ANCES, B., SABBAGH, M. & JOHNSON, S. 2020. Relationship of amyloid beta and neurofibrillary tau deposition in Neurodegeneration in Aging Down Syndrome (NiAD) study at baseline. *Alzheimer's & Dementia: Translational Research & Clinical Interventions,* 6**,** e12096.

TUDORASCU, D. L., ANDERSON, S. J., MINHAS, D. S., YU, Z., COMER, D., LAO, P., HARTLEY, S., LAYMON, C. M., SNITZ, B. E. & LOPRESTI, B. J. 2019. Comparison of longitudinal Aβ in nondemented elderly and Down syndrome. *Neurobiology of aging,* 73**,** 171-176.

VAN REEKUM, R., BLACK, S. E., CONN, D. & CLARKE, D. 1997. Cognition-enhancing drugs in dementia: a guide to the near future. *Can J Psychiatry,* 42 Suppl 1**,** 35s-50s.

VERA, E. & STUDER, L. 2015. When rejuvenation is a problem: challenges of modeling late-onset neurodegenerative disease. *Development,* 142**,** 3085-3089.

VIERBUCHEN, T., OSTERMEIER, A., PANG, Z. P., KOKUBU, Y., SÜDHOF, T. C. & WERNIG, M. 2010. Direct conversion of fibroblasts to functional neurons by defined factors. *Nature,* 463**,** 1035-1041.

VILLEMAGNE, V. L., ATAKA, S., MIZUNO, T., BROOKS, W. S., WADA, Y., KONDO, M., JONES, G., WATANABE, Y., MULLIGAN, R. & NAKAGAWA, M. 2009. High striatal amyloid β-peptide deposition across different autosomal Alzheimer disease mutation types. *Archives of neurology,* 66**,** 1537-1544.

VISSER, F. E., ALDENKAMP, A. P., VAN HUFFELEN, A. C., KUILMAN, M., OVERWEG, J. & VAN, W. J. 1997. Prospective study of the prevalence of Alzheimer-type dementia in institutionalized individuals with Down syndrome. *Am.J.Ment.Retard.,* 101**,** 400-412.

WANG, H. F. & LIU, F. C. 2001. Developmental restriction of the LIM homeodomain transcription factor Islet-1 expression to cholinergic neurons in the rat striatum. *Neuroscience,* 103**,** 999-1016.

WATANABE, K., KAMIYA, D., NISHIYAMA, A., KATAYAMA, T., NOZAKI, S., KAWASAKI, H., WATANABE, Y., MIZUSEKI, K. & SASAI, Y. 2005. Directed differentiation of telencephalic precursors from embryonic stem cells. *Nat Neurosci,* 8**,** 288-96.

WEATHERBEE, B. A. T., GANTNER, C. W., IWAMOTO-STOHL, L. K., DAZA, R. M., HAMAZAKI, N., SHENDURE, J. & ZERNICKA-GOETZ, M. 2023. Pluripotent stem cell-derived model of the post-implantation human embryo. *Nature,* 622**,** 584-593.

WEGIEL, J., FLORY, M., KUCHNA, I., NOWICKI, K., WEGIEL, J., MA, S. Y., ZHONG, N., BOBROWICZ, T. W., DE LEON, M., LAI, F., SILVERMAN, W. P. & WISNIEWSKI, T. 2022. Developmental deficits and staging of dynamics of age associated Alzheimer's disease neurodegeneration and neuronal loss in subjects with Down syndrome. *Acta Neuropathol Commun,* 10**,** 2.

WEICK, J. P., HELD, D. L., BONADURER, G. F., 3RD, DOERS, M. E., LIU, Y., MAGUIRE, C., CLARK, A., KNACKERT, J. A., MOLINAROLO, K., MUSSER, M., YAO, L., YIN,

Y., LU, J., ZHANG, X., ZHANG, S. C. & BHATTACHARYYA, A. 2013a. Deficits in human trisomy 21 iPSCs and neurons. *Proc Natl Acad Sci U S A,* 110**,** 9962-7.

WEICK, J. P., HELD, D. L., BONADURER, G. F., DOERS, M. E., LIU, Y., MAGUIRE, C., CLARK, A., KNACKERT, J. A., MOLINAROLO, K., MUSSER, M., YAO, L., YIN, Y. N., LU, J. F., ZHANG, X. Q., ZHANG, S. C. & BHATTACHARYYA, A. 2013b. Deficits in human trisomy 21 iPSCs and neurons. *Proceedings of the National Academy of Sciences of the United States of America,* 110**,** 9962-9967.

WEICK, J. P., KANG, H., BONADURER, G. F., 3RD & BHATTACHARYYA, A. 2016. Gene Expression Studies on Human Trisomy 21 iPSCs and Neurons: Towards Mechanisms Underlying Down's Syndrome and Early Alzheimer's Disease-Like Pathologies. *Methods Mol Biol,* 1303**,** 247-65.

WEINBERGER, N. M. 2007. Associative representational plasticity in the auditory cortex: a synthesis of two disciplines. *Learning & memory,* 14**,** 1-16.

WHITE, K. G. & RUSKE, A. C. 2002. Memory deficits in Alzheimer's disease: the encoding hypothesis and cholinergic function. *Psychon Bull Rev,* 9**,** 426-37.

WHITEHOUSE, P. J., PRICE, D. L., CLARK, A. W., COYLE, J. T. & DELONG, M. R. 1981. Alzheimer disease: evidence for selective loss of cholinergic neurons in the nucleus basalis. *Ann Neurol,* 10**,** 122-6.

WHITEHOUSE, P. J., PRICE, D. L., STRUBLE, R. G., CLARK, A. W., COYLE, J. T. & DELON, M. R. 1982. Alzheimer's disease and senile dementia: loss of neurons in the basal forebrain. *Science,* 215**,** 1237-9.

WILCOCK, D. M. 2012. Neuroinflammation in the Aging Down Syndrome Brain; Lessons from Alzheimer's Disease. *Current Gerontology and Geriatrics Research,* 2012**,** 170276.

WILLIAMS, R. H. & RIEDEMANN, T. 2021. Development, Diversity, and Death of MGE-Derived Cortical Interneurons. *International Journal of Molecular Sciences,* 22**,** 9297.

WISEMAN, F. K., AL-JANABI, T., HARDY, J., KARMILOFF-SMITH, A., NIZETIC, D., TYBULEWICZ, V. L. J., FISHER, E. M. C. & STRYDOM, A. 2015. A genetic cause of Alzheimer disease: mechanistic insights from Down syndrome. *Nature Reviews Neuroscience,* 16**,** 564-574.

WISEMAN, F. K., PULFORD, L. J., BARKUS, C., LIAO, F., PORTELIUS, E., WEBB, R., CHAVEZ-GUTIERREZ, L., CLEVERLEY, K., NOY, S., SHEPPARD, O., COLLINS, T., POWELL, C., SARELL, C. J., RICKMAN, M., CHOONG, X., TOSH, J. L., SIGANPORIA, C., WHITTAKER, H. T., STEWART, F., SZARUGA, M., MURPHY, M. P., BLENNOW, K., DE STROOPER, B., ZETTERBERG, H., BANNERMAN, D., HOLTZMAN, D. M., TYBULEWICZ, V. L. J., FISHER, E. M. C. & LONDON DOWN SYNDROME, C. 2018. Trisomy of human chromosome 21 enhances amyloid-beta deposition independently of an extra copy of APP. *Brain,* 141**,** 2457-2474.

WISNIEWSKI, K., DALTON, A., MCLACHLAN, D. C., WEN, G. & WISNIEWSKI, H. 1985. Alzheimer's disease in Down's syndrome: clinicopathologic studies. *Neurology,* 35**,** 957-957.

WISNIEWSKI, K. E., LAURE-KAMIONOWSKA, M. & WISNIEWSKI, H. M. 1984. Evidence of arrest of neurogenesis and synaptogenesis in brains of patients with Down's syndrome. *N.Engl.J.Med.,* 311**,** 1187-1188.

WOOLF, N. J. 1991. Cholinergic systems in mammalian brain and spinal cord. *Prog Neurobiol,* 37**,** 475-524.

WU, H., WILLIAMS, J. & NATHANS, J. 2014. Complete morphologies of basal forebrain cholinergic neurons in the mouse. 3.

XIE, Z., BAILEY, A., KULESHOV, M. V., CLARKE, D. J. B., EVANGELISTA, J. E., JENKINS, S. L., LACHMANN, A., WOJCIECHOWICZ, M. L., KROPIWNICKI, E., JAGODNIK, K. M., JEON, M. & MA'AYAN, A. 2021. Gene Set Knowledge Discovery with Enrichr. *Current Protocols,* 1**,** e90.

XU, L., HUO, H.-Q., LU, K.-Q., TANG, X.-Y., HONG, Y., HAN, X., FU, Z.-X., FANG, K.-H., XU, M., GUO, X. & LIU, Y. 2022. Abnormal mitochondria in Down syndrome iPSC-derived GABAergic interneurons and organoids. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease,* 1868**,** 166388.

XU, Q., GUO, L., MOORE, H., WACLAW, R. R., CAMPBELL, K. & ANDERSON, S. A. 2010. Sonic hedgehog signaling confers ventral telencephalic progenitors with distinct cortical interneuron fates. *Neuron,* 65**,** 328-40.

XU, Q., TAM, M. & ANDERSON, S. A. 2008. Fate mapping Nkx2.1-lineage cells in the mouse telencephalon. *J Comp Neurol,* 506**,** 16-29.

XU, Q., WONDERS, C. P. & ANDERSON, S. A. 2005. Sonic hedgehog maintains the identity of cortical interneuron progenitors in the ventral telencephalon. *Development,* 132**,** 4987-98.

XU, W., FANG, F., DING, J. & WU, C. 2018. Dysregulation of Rab5-mediated endocytic pathways in Alzheimer's disease. *Traffic,* 19**,** 253-262.

XU, W., WEISSMILLER, A. M., WHITE, J. A., FANG, F., WANG, X. Y., WU, Y. W., PEARN, M. L., ZHAO, X. B., SAWA, M., CHEN, S. D., GUNAWARDENA, S., DING, J. Q., MOBLEY, W. C. & WU, C. B. 2016. Amyloid precursor protein-mediated endocytic pathway disruption induces axonal dysfunction and neurodegeneration. *Journal of Clinical Investigation,* 126**,** 1815-1833.

YAN, R. & VASSAR, R. 2014. Targeting the β secretase BACE1 for Alzheimer's disease therapy. *The Lancet Neurology,* 13**,** 319-329.

YANKNER, B. A., DUFFY, L. K. & KIRSCHNER, D. A. 1990. NEUROTROPHIC AND NEUROTOXIC EFFECTS OF AMYLOID BETA-PROTEIN - REVERSAL BY TACHYKININ NEUROPEPTIDES. *Science,* 250**,** 279-282.

YATES, C. M., SIMPSON, J., MALONEY, A. F. J., GORDON, A. & REID, A. H. 1980. ALZHEIMER-LIKE CHOLINERGIC DEFICIENCY IN DOWN SYNDROME. *Lancet,* 2**,** 979-979.

YIANNOPOULOU, K. G. & PAPAGEORGIOU, S. G. 2020. Current and Future Treatments in Alzheimer Disease: An Update. *Journal of Central Nervous System Disease,* 12**,** 1179573520907397.

YU, J., VODYANIK, M. A., SMUGA-OTTO, K., ANTOSIEWICZ-BOURGET, J., FRANE, J. L., TIAN, S., NIE, J., JONSDOTTIR, G. A., RUOTTI, V., STEWART, R., SLUKVIN, I. I. & THOMSON, J. A. 2007. Induced pluripotent stem cell lines derived from human somatic cells. *Science,* 318**,** 1917-1920.

YU, T., LI, Z. Y., JIA, Z. P., CLAPCOTE, S. J., LIU, C. H., LI, S. M., ASRAR, S., PAO, A., CHEN, R. Q., FAN, N., CARATTINI-RIVERA, S., BECHARD, A. R., SPRING, S. S., HENKELMAN, R. M., STOICA, G., MATSUI, S. I., NOWAK, N. J., RODER, J. C., CHEN, C., BRADLEY, A. & YU, Y. E. 2010. A mouse model of Down syndrome trisomic for all human chromosome 21 syntenic regions. *Human Molecular Genetics,* 19**,** 2780-2791.

YUE, W., LI, Y. Y., ZHANG, T., JIANG, M., QIAN, Y., ZHANG, M., SHENG, N. Y., FENG, S., TANG, K., YU, X., SHU, Y. S., YUE, C. M. & JING, N. H. 2015. ESC-Derived Basal Forebrain Cholinergic Neurons Ameliorate the Cognitive Symptoms Associated with Alzheimer's Disease in Mouse Models. *Stem Cell Reports,* 5**,** 776-790.

ZABORSZKY, L., CSORDAS, A., MOSCA, K., KIM, J., GIELOW, M. R., VADASZ, C. & NADASDY, Z. 2015. Neurons in the Basal Forebrain Project to the Cortex in a Complex Topographic Organization that Reflects Corticocortical Connectivity Patterns: An Experimental Study Based on Retrograde Tracing and 3D Reconstruction. *Cerebral Cortex,* 25**,** 118-137.

ZABORSZKY, L., ROSIN, D. L. & KISS, J. 2004. Alpha-adrenergic receptor (alpha(2 A)) is colocalized in basal forebrain cholinergic neurons: a light and electron microscopic double immunolabeling study. *J Neurocytol,* 33**,** 265-76.

ZAMMIT, M. D., LAYMON, C. M., BETTHAUSER, T. J., CODY, K. A., TUDORASCU, D. L., MINHAS, D. S., SABBAGH, M. N., JOHNSON, S. C., ZAMAN, S. H. & MATHIS, C. A. 2020a. Amyloid accumulation in Down syndrome measured with amyloid load. *Alzheimer's & Dementia: Diagnosis, Assessment & Disease Monitoring,* 12**,** e12020.

ZAMMIT, M. D., LAYMON, C. M., TUDORASCU, D. L., HARTLEY, S. L., PIRO-GAMBETTI, B., JOHNSON, S. C., STONE, C. K., MATHIS, C. A., ZAMAN, S. H. & KLUNK, W. E. 2020b. Patterns of glucose hypometabolism in Down syndrome resemble sporadic Alzheimer's disease except for the putamen. *Alzheimer's & Dementia: Diagnosis, Assessment & Disease Monitoring,* 12**,** e12138.

ZAMMIT, M. D., TUDORASCU, D. L., LAYMON, C. M., HARTLEY, S. L., ZAMAN, S. H., ANCES, B. M., JOHNSON, S. C., STONE, C. K., MATHIS, C. A. & KLUNK, W. E. 2021. PET measurement of longitudinal amyloid load identifies the earliest stages of amyloid-beta accumulation during Alzheimer's disease progression in Down syndrome. *Neuroimage,* 228**,** 117728.

ZHANG, K., FISHEL BEN KENAN, R., OSAKADA, Y., XU, W., SINIT, R. S., CHEN, L., ZHAO, X., CHEN, J.-Y., CUI, B. & WU, C. 2013. Defective Axonal Transport of Rab7 GTPase Results in Dysregulated Trophic Signaling. *The Journal of Neuroscience,* 33**,** 7451-7462.

ZHANG, S. C., WERNIG, M., DUNCAN, I. D., BRUSTLE, O. & THOMSON, J. A. 2001. In vitro differentiation of transplantable neural precursors from human embryonic stem cells. *Nat Biotechnol,* 19**,** 1129-33.

ZHANG, Y., CHEN, H., LI, R., STERLING, K. & SONG, W. 2023. Amyloid β-based therapy for Alzheimer's disease: challenges, successes and future. *Signal Transduction and Targeted Therapy,* 8**,** 248.

ZHAO, X. & BHATTACHARYYA, A. 2018. Human Models Are Needed for Studying Human Neurodevelopmental Disorders. *Am J Hum Genet,* 103**,** 829-857.

ZHAO, Y., MARÍN, O., HERMESZ, E., POWELL, A., FLAMES, N., PALKOVITS, M., RUBENSTEIN, J. L. & WESTPHAL, H. 2003. The LIM-homeobox gene Lhx8 is required for the development of many cholinergic neurons in the mouse forebrain. *Proc Natl Acad Sci U S A,* 100**,** 9005-10.

ZHENG, G. X., TERRY, J. M., BELGRADER, P., RYVKIN, P., BENT, Z. W., WILSON, R., ZIRALDO, S. B., WHEELER, T. D., MCDERMOTT, G. P. & ZHU, J. 2017. Massively parallel digital transcriptional profiling of single cells. *Nature communications,* 8**,** 14049.

ZHENG, X., BOYER, L., JIN, M., MERTENS, J., KIM, Y., MA, L., MA, L., HAMM, M., GAGE, F. H. & HUNTER, T. 2016. Metabolic reprogramming during neuronal differentiation from aerobic glycolysis to neuronal oxidative phosphorylation. *Elife,* 5.

ZHONG, Z., OHNMACHT, J., REIMER, M. M., BACH, I., BECKER, T. & BECKER, C. G. 2012. Chondrolectin mediates growth cone interactions of motor axons with an intermediate target. *J Neurosci,* 32**,** 4426-39.

ZHOU, X., CHEN, X., HONG, T., ZHANG, M., CAI, Y. & CUI, L. 2022. TTC3-Mediated Protein Quality Control, A Potential Mechanism for Cognitive Impairment. *Cell Mol Neurobiol,* 42**,** 1659-1669.

ZHOU, Y., SUN, Y., MA, Q.-H. & LIU, Y. 2018. Alzheimer's disease: Amyloid-based pathogenesis and potential therapies. *Cell stress,* 2**,** 150.

ZHOU, Y., TAO, L. & ZHU, Y. 2023. TempShift Reveals the Sequential Development of Human Neocortex and Skewed Developmental Timing of Down Syndrome Brains. *Brain Sci,* 13.

ZIGMAN, W. B., SCHUPF, N., SERSEN, E. & SILVERMAN, W. 1996. Prevalence of dementia in adults with and without Down syndrome. *Am.J.Ment.Retard.,* 100**,** 403-412.