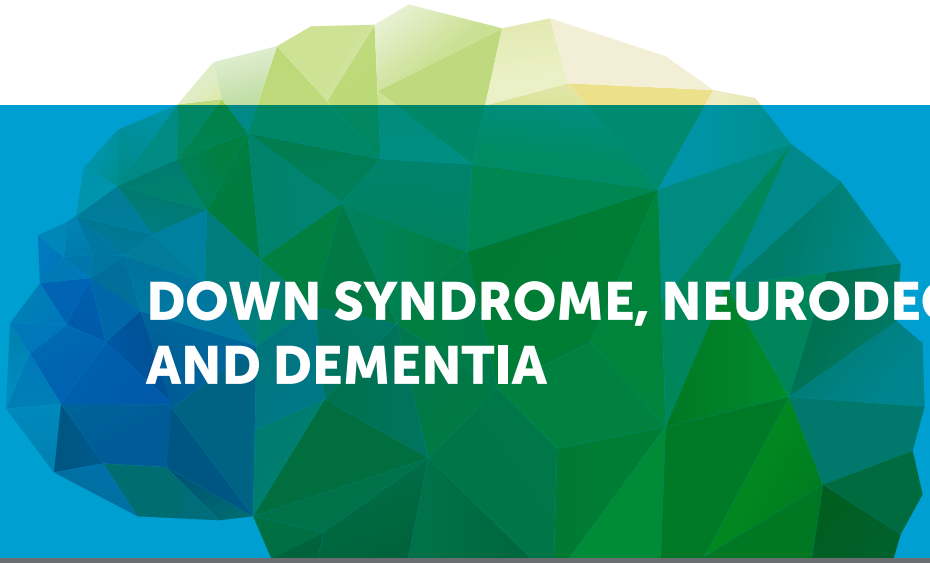

DOWN SYNDROME, NEURODEGENERATION AND DEMENTIA

EDITED BY: Sylvia Eva Perez, Tao Ma, Elliott Jay Mufson, Stephen D. Ginsberg and Aurélie Ledreux

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DOWN SYNDROME, NEURODEGENERATION AND DEMENTIA

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Editorial: Down Syndrome, Neurodegeneration and Dementia

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Editorial on the Research Topic

Down Syndrome, Neurodegeneration and Dementia

The Cornish physician John L. Down published a paper entitled "Observations on an ethnic classification of idiots" (Down, 1866) (**Figure 1**), describing a condition referred to as a "mongoloid idiot." Interestingly, Down's grandson was born with this condition (**Figure 1**) (Salehi et al., 2016). Down assumed that parental tuberculosis caused this disorder (Van Robays, 2016). However, almost a century later, genetic analysis by Lejeune, Gautier and Turpin (Lejeune et al., 1959) revealed that this syndrome was due to an extra copy of chromosome 21 (HSA21) (**Figure 1**), which encodes the gene for amyloid-beta precursor protein (APP). In 1965, the World Health Organization confirmed the eponym for this disorder as Down syndrome (DS). The discovery of the gene that encodes the APP protein, which includes the beta-amyloid (Aβ) peptide, and that resides on chromosome 21 was first reported by Goldgaber (Goldgaber et al., 1987) followed by other published works (Kang et al., 1987; Robakis et al., 1987; Watkins et al., 1987; Korenberg et al., 1989). Trisomy 21 leads to an overproduction of the Aβ peptide associated with DS (Glenner and Wong, 1984), AD (Wisniewski et al., 1988), and familiar AD (FAD) (Teller et al., 1996; Russo et al., 1997; Mori et al., 2002). It is interesting to note that several genes on chromosome 21 have been associated with cognitive dysfunction in DS, however, the APP gene alone is necessary and sufficient to cause dementia (Doran et al., 2017). Recently, it was reported that DS affects approximately 200,000 people in the US and 5–8 million worldwide (de Graaf et al., 2017). Interestingly, there is an age-associated clinical and pathological coexistence between DS and AD, which is a major public health issue. Life expectancy of people with DS has increased dramatically over the past decades (from 25 years in the 1980s to 60+ years currently) and consequently age-related cognitive syndromes have also increased (Ruparelia et al., 2013; Godfrey and Lee, 2018). However, the neurobiology underlying the onset of dementia in individuals with DS remains a complex question. Individuals with DS develop selective neuronal degeneration, synaptic loss, neurofibrillary tangles, and Aβ plaques similar to AD (Mirra et al., 1991; Hyman and Trojanowski, 1997) by the fourth decade of life (Mann et al., 1989; Hartley et al., 2015) and is now recognized as a genetically-determined form of AD (Fortea et al., 2020).

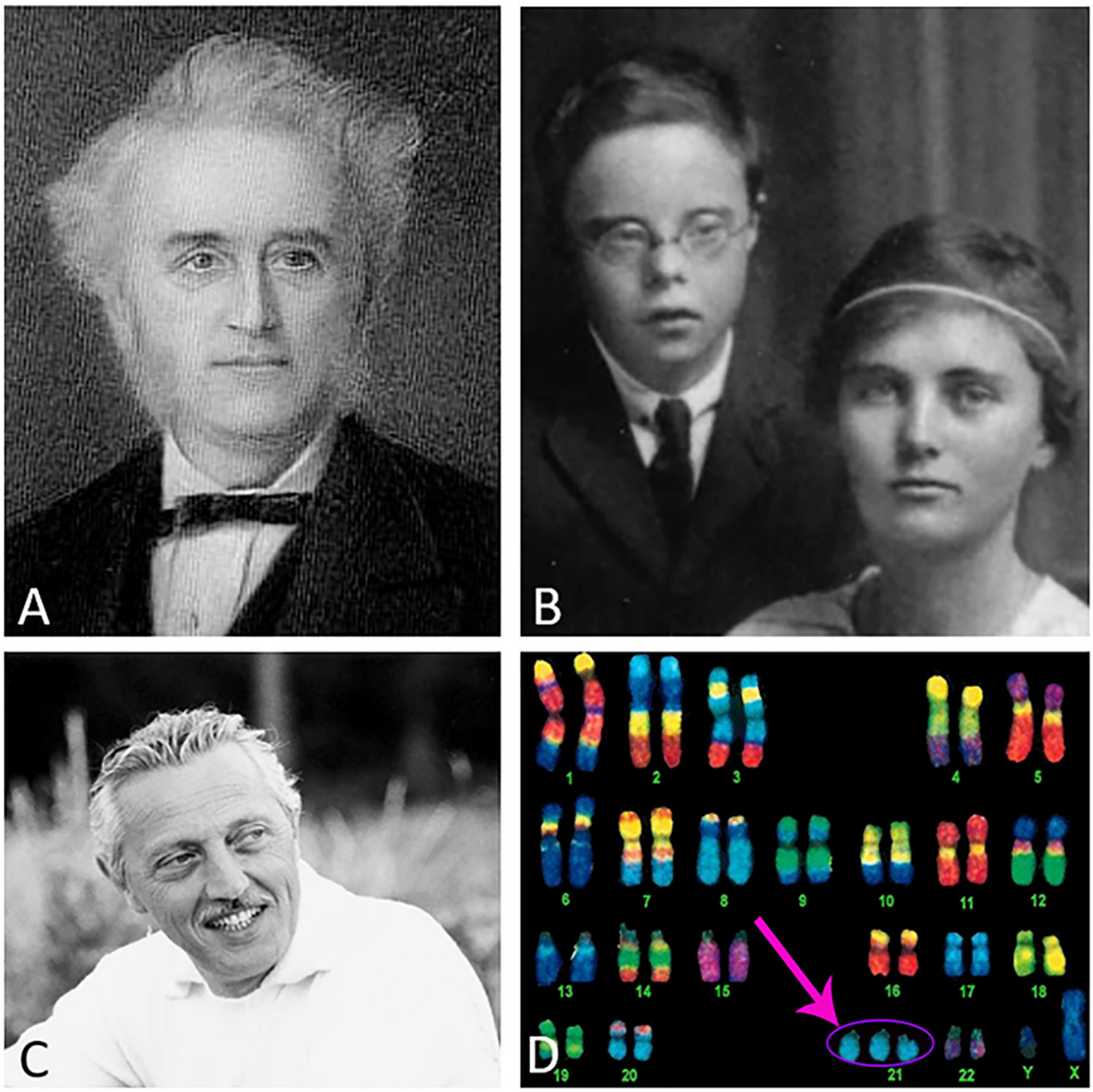

FIGURE 1 | Dr. Down **(A)**, Down's grandson and daughter **(B)**, Dr. Lejeune **(C)**, and an image of a Down syndrome karyotype indicating the extra copy of chromosome 21 (pink arrow) **(D)**. Image credits: **(A)** Wikipedia, **(B)** photo courtesy of Global Down Syndrome Foundation, **(C)** reproduced with permission from the Jerome Lejeune Foundation, and **(D)** @prayersforbabyfinn webpage.

Approximately 70% of people with DS >50 years of age display dementia, which may be an underestimate. Despite DS being the largest group of individuals with early-onset AD, there is a lack of knowledge defining the mechanisms driving neuronal and functional dysfunction in both disorders, impeding drug discovery. Importantly, the prevalence of AD in DS makes it possible to enroll this population in clinical trials. Although not totally representative of either disorder, mouse models recapitulate key aspects of DS and AD, enabling the assessment of pathophysiological mechanisms (Reeves et al., 1995; Li et al., 2007; Haydar and Reeves, 2012). The current Research Topic "Down syndrome, Neurodegeneration and Dementia" highlights basic and translational research in DS. In total, seven manuscripts evaluated human DS and three reports studied murine models of DS and related AD pathobiology.

Chen et al. discuss products of triplicated genes on HSA21 that may modify the effect of APP in DS related to endosomal-lysosomal, neurotrophin, axonal transport, and immunological

cellular systems that affect people with DS that go on to contract Covid-19.

Pivtoraiko et al. discuss the interaction between Pittsburgh Compound B (PiB), or related amyloid binding radiopharmaceuticals for positron emission tomography (PET) imaging, with different unmodified Aβ forms or post-translationally truncated and pyroglutamate-modified Aβ in adults with DS and AD. Despite the distinct molecular profile of Aβ forms and greater vascular amyloidosis in DS, cortical ^{3}H-PiB binding does not distinguish between groups at an advanced level of amyloid plaque pathology suggesting differences in pathobiological mechanism(s) driving dementia.

Ahmed et al. suggest that the innate immune system activator granulocyte-macrophage colony-stimulating factor (GM-CSF) may have a therapeutic and/or compensatory action in animal models of DS, AD, and normal aging. They argue that in AD clinical trials activating the innate immune system may have paradoxical effects, and that inflammation may be therapeutic rather than deleterious.

Martinez et al. review the role of basal forebrain cholinergic (BFC) neuronal function and degeneration in AD and DS and identify under-studied aspects of BFC neuronal biology. Cuello and coworkers (Do Carmo et al.) review mechanisms underlying the compromise of the neurotrophin, nerve growth factor (NGF) in AD and DS. Similarities between dysfunction in the NGF neurotrophic system suggests that drugs related to the preservation of this neurotrophic pathway are treatment approaches for both DS and AD.

The Mufson group (Miguel et al.) examined the effect of trisomy on amyloid, Purkinje cells (PC), and interneurons within the cerebellum in DS. Their findings suggest that disturbances in calcium binding proteins play a critical role in cerebellar neuronal circuit dysfunction in adults with DS. The data suggests that drugs targeting specific calcium binding proteins are a novel target to prevent cerebellar cellular degeneration, which could impact cognition in DS.

Wang et al. explored sex-related genetic heterogeneity in AD by investigating single nucleotide polymorphism (SNP) heritability, genetic correlation, as well as SNP- and gene-based genome-wide analyses. The authors indicate an overall similar genetic architecture of AD in both sexes at the genome-wide averaged level and that clinically observed sex differences arise from sex-specific variants. This observation is important for the development of personalize medicine.

The article by the Ginsberg laboratory (Alldred et al.) investigated dysregulation of genes and encoded proteins of the oxidative phosphorylation pathway within the basocortical projection system in young Ts65Dn mice. The authors suggest that dysregulation within mitochondrial oxidative phosphorylation complexes is an early marker of basocortical degeneration in DS. These findings indicate a crucial role for alterations of oxidative gene expression as a potential avenue for future treatment approaches for DS with translation to AD.

The Velazquez group (Winslow et al.) discusses the use of the novel IntelliCage behavioral testing apparatus to overcome pervasive animal handling issues that occur during cognitive testing using the well-established 3xTg-AD animal model. The authors demonstrate deficits in cognition in the 3xTg-AD mouse and provide important factors to consider when testing models of AD and DS in the IntelliCage. These findings suggest that this novel technology is an important new tool for the investigation of cognitive deficits in animal models of dementia.

Strupp and coworkers (Powers et al.) present new evidence that dietary maternal choline supplementation during pregnancy and lactation has beneficial effects on cognition in young and old Ts65Dn mice throughout life, suggesting that this nutritional supplement would have population-wide benefits and provide an early intervention for DS fetuses.

A general comment about the Research Topic: Down syndrome, Neurodegeneration and Dementia. It is difficult to include all aspects of basic, translational, and clinical research related to DS in the context of a series of a dedicated papers. Rather, a tacit goal of the Research Topic in *Frontiers in Aging Neuroscience* is to increase overall interest in this underserved area of research and bring new investigators from other fields that will use *in vivo* and *in vitro* models of DS and AD. We also encourage studies using clinically and neuropathologically well-characterized tissue from human DS brain repositories to further provide therapeutic development that will assist this very special population of individuals as well as drug and treatment discovery for AD dementia and related disorders.

AUTHOR CONTRIBUTIONS

EM drafted the manuscript. SG, TM, AL, and SP edited the manuscript. All authors take responsibility for the integrity of the data and the accuracy of the data presented in the article. All authors contributed to the article and approved the submitted version.

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Cerebellar Calcium-Binding Protein and Neurotrophin Receptor Defects in Down Syndrome and Alzheimer's Disease

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Cerebellar hypoplasia is a major characteristic of the Down syndrome (DS) brain. However, the consequences of trisomy upon cerebellar Purkinje cells (PC) and interneurons in DS are unclear. The present study performed a quantitative and qualitative analysis of cerebellar neurons immunostained with antibodies against calbindin D-28k (Calb), parvalbumin (Parv), and calretinin (Calr), phosphorylated and non-phosphorylated intermediate neurofilaments (SMI-34 and SMI-32), and high (TrkA) and low ($p75^{NTR}$) affinity nerve growth factor (NGF) receptors as well as tau and amyloid in DS ($n = 12$), Alzheimer's disease (AD) ($n = 10$), and healthy non-dementia control (HC) ($n = 8$) cases. Our findings revealed higher $A\beta_{42}$ plaque load in DS compared to AD and HC but no differences in APP/Aβ plaque load between HC, AD, and DS. The cerebellar cortex neither displayed $A\beta_{40}$ containing plaques nor pathologic phosphorylated tau in any of the cases examined. The number and optical density (OD) measurements of Calb immunoreactive (-ir) PC soma and dendrites were similar between groups, while the number of PCs positive for Parv and SMI-32 were significantly reduced in AD and DS compared to HC. By contrast, the number of SMI-34-ir PC dystrophic axonal swellings, termed torpedoes, was significantly greater in AD compared to DS. No differences in SMI-32- and Parv-ir PC OD measurements were observed between groups. Conversely, total number of Parv- (stellate/basket) and Calr (Lugaro, brush, and Golgi)-positive interneurons were significantly reduced in DS compared to AD and HC. A strong negative correlation was found between counts for Parv-ir interneurons, Calr-ir Golgi and brush cells, and $A\beta_{42}$ plaque load. Number of TrkA and $p75^{NTR}$ positive PCs were reduced in AD compared to HC. These findings suggest that disturbances in calcium binding proteins play a critical role in cerebellar neuronal dysfunction in adults with DS.

Keywords: Down syndrome, Alzheimer's disease, cerebellum, calcium binding proteins, Purkinje cells, nerve growth factor receptors, amyloid, tau

INTRODUCTION

Down Syndrome (DS), or trisomy 21, is a genetic disorder caused by an extra copy of chromosome 21 (HSA21), which is characterized by developmental delay and intellectual disability (Potier and Reeves, 2016). By the age of 40, people with DS develop a pathological profile consisting of amyloid plaques and tau containing neurofibrillary tangles (NFTs) within the neo and limbic cortex similar to Alzheimer's disease (AD) (Wisniewski et al., 1985; Davidson et al., 2018) as well as reduction in brain volume (de la Monte and Hedley-Whyte, 1990; Weis et al., 1991; Kesslak et al., 1994; Aylward et al., 1999; Teipel et al., 2003; Edgin et al., 2015; Cipriani et al., 2018), including the cerebellum (Weis et al., 1991; Jernigan et al., 1993). In DS, cerebellar hypoplasia has been attributed to developmental disturbances in neurogenesis (Guidi et al., 2011) that contribute to deficits in cognition (Jernigan et al., 1993; Pinter et al., 2001; Pennington et al., 2003; Carr, 2005; Lott and Dierssen, 2010) and motor function (Spanò et al., 1999; Lee et al., 2020). Although cerebellar atrophy and beta amyloid plaques, but not NFTs, develop during aging in DS (Mann and Jones, 1990; Cole et al., 1993; Li et al., 1994), the cellular pathobiology underlying cerebellar dysfunction remains an under-investigated area.

The cerebellum has a well-established role in the coordination of movements (Houk and Miller, 2001; Morton and Bastian, 2004; Koziol et al., 2014) but also plays a role in higher order functions, including emotion, language, and cognition (Gordon, 1996; de Smet et al., 2007, 2013; Turner et al., 2007). Morphologically, the cerebellum is a tri-laminar structure comprised of a superficial molecular layer mainly containing basket and stellate interneurons, a middle cell layer consisting of a monolayer of Purkinje cells (PCs) and a deep granular cell layer containing small excitatory granule cells (e.g., brush cells) and inhibitory (Golgi and Lugaro) interneurons (Roostaei et al., 2014). Cerebellar gamma-aminobutyric acid (GABA) inhibitory PCs provide the major cerebellar output to the deep cerebellar and vestibular nuclei (Herndon, 1963; Delgado-Garcia, 2001; Houk and Miller, 2001). Cerebellar excitatory inputs (Wadiche and Jahr, 2001; Bagnall and du Lac, 2006; Arenz et al., 2009; Kurtaj et al., 2013; Roostaei et al., 2014) that arise from the brainstem/spinal cord and inferior olivary nuclei course within the mossy fiber-granule cell parallel and climbing fibers, respectively (Hoxha et al., 2018). PCs contain the calcium-binding proteins (CBPs) calbindin D-28k (Calb) and parvalbumin (Parv), while calretinin (Calr) is found exclusively in cells within the cerebellar granule cell layer (Bastianelli, 2003). CBPs maintain intracellular calcium homeostasis, which play a key role in synaptic function (Iacopino and Christakos, 1990; Airaksinen et al., 1997; Caillard et al., 2000; Bastianelli, 2003; Gattoni and Bernocchi, 2019). Disruption of cellular homeostasis, due to an increase of free cytoplasmic calcium, induces neuronal apoptosis (Orrenius et al., 2003). Although it was hypothesized that calcium dysregulation plays a major role in the cellular pathogenesis of AD (Khachaturian, 1994), less is known about its actions in DS.

Reduction in Calb and Parv containing neurons has been reported in the frontal and temporal cortex of adults with DS (Kobayashi et al., 1990). Interestingly, knockout of Calb and Parv in PCs impairs motor coordination and sensory processing (Airaksinen et al., 1997; Barski et al., 2003) with the most severe deficits occurring when both proteins are deleted in rodents (Farré-Castany et al., 2007). Furthermore, Calr null mice display impaired granule cells and PC function (Schiffmann et al., 1999), while the induction of Calr restores normal cerebellar function (Bearzatto et al., 2006). These data suggest that disturbances in CBPs within the neurons of cerebellar cortex contribute to motor and cognitive impairment in DS. However, the effects of trisomy upon CBP containing neurons in the cerebellum of individuals with DS remain unclear.

In addition to CBPs, PCs also contain the cognate receptors TrkA and $p75^{NTR}$ for the neuronal survival protein, nerve growth factor (NGF) (Mufson et al., 1991; Savaskan et al., 2000). Although these receptors are constitutively expressed during early cerebellar development through adulthood (Muragaki et al., 1995; Roux and Barker, 2002; Quartu et al., 2003a,b; Florez-McClure et al., 2004; Schor, 2005; Lotta et al., 2014), their role in PC pathology in DS is poorly defined. Numerous studies indicate that NGF receptors play a key role in cholinergic basal forebrain neuron dysfunction in AD and DS (Sendera et al., 2000; Mufson et al., 2019). However, the effect of trisomy upon TrkA and $p75^{NTR}$ and their relationship with CBPs in PCs in DS remains to be investigated. Therefore, the present study examined CBPs and NGF receptors in the cerebellar cortex in DS and AD compared to healthy non-dementia subjects (HC) using quantitative immunohistochemistry, densitometry, and morphometry.

MATERIALS AND METHODS

Subjects

Cerebellar cortex from a total of 30 adults (44–98 years of age) who died with an ante-mortem clinical diagnosis of AD, DS, or HC was obtained from the Rush University Department of Pathology, Chicago, IL (8 HC, 10 AD, and 5 DS cases), University of California at Irvine Alzheimer's Disease Research Center (UCI ADRC; 6 DS cases), and the Barrow Neurological Institute at St. Joseph's Hospital and Medical Center, Phoenix, AZ, USA (BNI; 1 DS case). Of the 12 DS cases, 9 had dementia (DSD+), and 3 did not (DSD+) and three were non-demented (DSD–). Tissue collection and handling conformed to the guidelines of each respective Institutional Review Board (IRB) protocol. DS diagnosis was confirmed by the presence of an extra copy of HSA21 using fluorescence *in situ* hybridization and/or chromosome karyotyping.

Dementia status was determined as previously reported (Perez et al., 2019). Briefly, the clinical status of the UCI ADRC DS participants was determined in accordance with International Classification of Diseases and Related Health Problems-Tenth Revision (ICD-10) and Dementia Questionnaire for Mentally Retarded Persons (DMR-IV-TR) criteria (Sheehan et al., 2015). All UCI ADRC and the BNI DS cases were participants in longitudinal research protocols prior to death. Assessments included physical and neurological exams and a history

obtained from both the participant and a reliable caregiver. Standardized direct and indirect cognitive and behavioral assessments were also completed. The diagnosis of dementia required deficits in two or more areas of cognitive functioning and progressive worsening of cognitive performance compared to the baseline performance of an individual. Cases with cognitive decline due to confounding factors that may mimic dementia (e.g., depression, sensory deficits, and hypothyroidism) were eliminated. Premorbid-intelligence quotient (IQ) was also determined in all the UCI DS cases. Determination of clinical status of the Rush cases was performed by a neurologist trained in gerontology together with discussions with a caregiver. Human Research Committees of Rush University Medical Center, University of California at Irvine, and Barrow Neurological Institute approved this study. **Supplementary Table 1** details the clinical, demographic, and neuropathological features and tissue source of the cases used in this study.

Neuropathological Evaluation

Since virtually all of the tissue utilized in this study was obtained from archival cases collected prior to the establishment of the National Alzheimer's Coordinating Center, National Institute on Aging (NIA)-Reagan criteria (Newell et al., 1999), Consortium to Establish a Registry for Alzheimer's Disease (CERAD) (Mirra et al., 1991), and Thal amyloid staging (Thal et al., 2002) were not available. Neuropathological diagnosis was based on Braak staging of NFTs (Braak and Braak, 1991). None of the cases examined were treated with acetylcholinesterase inhibitors.

Tissue Processing

The cerebellum was immersion fixed in either 4% paraformaldehyde or 10% formalin for 3–10 days, cut on a sliding freezing microtome at 40 μm thickness, and stored in cryoprotectant (40% phosphate buffer pH7.4, 30% glycerol, and 30% ethylene glycol) at −20°C prior to processing as previously reported (Perez et al., 2019). All free-floating sections were mounted on positive charged slides and air-dried overnight prior to the histochemical [hematoxylin and eosin (H&E) and cresyl violet] and immunohistochemical procedures.

Immunohistochemistry

Antibody characteristics, dilution, and the commercial company from which each was purchased are shown in **Table 1**. Before immunostaining, cerebellar sections were pretreated for antigen retrieval with boiling citric acid (pH 6) for 10 min for Calb, Parv, Calr, SMI-32, SMI-34 and 15 min for TrkA and $p75^{NTR}$. Antigen retrieval for the 6E10, $A\beta_{40}$, and $A\beta_{42}$ antibodies consisted of placing sections in 88% formic acid at room temperature (RT) for 10 min. Sections were then washed in Tris-buffered saline (TBS, pH 7.4) followed by incubation in 0.1 M sodium metaperiodate (Sigma-Aldrich, St. Louis, MO, USA) to inactivate endogenous peroxidases, permeabilized in TBS containing 0.25% Triton X-100 (Thermo Fisher Scientific, Waltham, MA, USA) and blocked in TBS/0.25% Triton containing 3% goat serum for 1 h. Sections were incubated with primary antibodies overnight at RT in TBS containing 0.25% Triton X-100 and 1% goat serum. The next day, after three washes with TBS/1% goat serum, sections were incubated with affinity-purified goat anti-mouse or goat anti-rabbit biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA, USA) for 1 h at RT. After washes in TBS, sections were incubated in Vectastain Elite ABC kit (Vector Laboratories) for 1 h at RT and developed in acetate-imidazole buffer containing the chromogen 0.05% 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich). Immunoreactivity for TrkA and $p75^{NTR}$ was enhanced using a solution consisting of DAB and nickel sulfate (0.5–1.0%). The reaction was terminated in acetate-imidazole buffer (pH 7.4), and sections were dehydrated in graded alcohols, cleared in xylenes, and cover-slipped with DPX mounting medium (Electron Microscopy Sciences, Hatfield, PA, USA). Cytochemical controls consisted of the omission of primary antibodies, which resulted in no detectable immunoreactivity (see **Supplementary Figures 1A–D**). In addition, a series of control experiments were performed to demonstrate that the TrkA antibody used in this study does not immunostain neurons containing TrkB. First, as a positive control for both TrkA and the $p75^{NTR}$ immunolabeling, we stained sections containing the cholinergic neurons within the nucleus basalis of Meynert (nbM), which display both of these proteins (Mufson et al., 1989; Perez et al., 2012), obtained from a female 93-year-old HC and a female 94-year-old AD case, respectively. **Supplementary Figures 1E,F** show positive cellular nbM reactivity for each antibody. Secondly, sections containing neurons located within the oculomotor nucleus (cranial nerve III) and the substantia nigra (SN) of a male 51-year-old HC case were immunostained using the current TrkA antibody. Although oculomotor neurons also displayed TrkA immunoreactivity, SN neurons, which contain TrkB (Jin, 2020), were TrkA (Sobreviela et al., 1994) immunonegative (**Supplementary Figures 1G,H**). Some sections were counterstained with Gill's hematoxylin or cresyl violet for laminar identification.

Immunofluorescence

To evaluate the relationship between Calb, Parv, and SMI-32, cerebellar sections were mounted onto positive charged slides, pretreated with boiling citric acid (pH6) for 10 min for antigen retrieval, washed with TBS, blocked in a TBS/0.5%Triton solution containing 3% donkey serum, and dual-labeled with rabbit anti-Calb and mouse anti-Parv or mouse anti-SMI-32. Sections were incubated overnight for Calb at RT washed with TBS/1% donkey serum and incubated in Cy2-conjugated donkey anti-rabbit immunoglobulin G (IgG) secondary antibody (1:200, Jackson ImmunoResearch, West Grove, PA, USA) for 1 h. After several washes with TBS/1% donkey serum, sections were incubated overnight using either anti-Parv or anti-SMI-32 at RT and then placed in Cy3-conjugated donkey anti-mouse IgG secondary antibody for 1 h (1:200, Jackson ImmunoResearch). Following development, sections were washed in TBS, dehydrated in graded alcohols, cleared in xylenes, and cover-slipped with DPX mounting medium. Fluorescence was visualized with the aid of a Revolve Fluorescent Microscope (Echo Laboratories, San Diego, CA, USA) with excitation filters at wavelengths 489 and 555 nm for Cy2 and Cy3, respectively.

TABLE 1 | Antibody characteristics.

Antigen	Primary antibody	Dilution IH (IF)	Company: Catalog#	Secondary antibody Company: Catalog#
Tau (AT8)	Mouse monoclonal to phosphorylated-tau (Ser202/Thr205)	1:1,000	Invitrogen: MN1020	Biotinylated goat anti-mouse IgG Vector Laboratories: BA9200
APP/Aβ	Mouse monoclonal to residues 1–16 of N-terminus human Aβ (6E10)	1:1,000	Biolegend: 803002	
$Aβ_{40}$	Rabbit polyclonal to 7 aa peptide sequence from C-terminus of human Aβ1–40	1:1,000	Millipore: AB5074P	Biotinylated goat anti-rabbit IgG Vector Laboratories: BA1000 *Cy2-Donkey anti-rabbit IgG Jackson ImmunoResearch Laboratories: 711225152
$Aβ_{42}$	Rabbit polyclonal to 6 aa peptide sequence from C-terminus of human Aβ1–42	1:1,000	Millipore: AB5078P	
*Calbindin D-28k	Rabbit polyclonal to 28 kD calcium-binding protein	1:15,000 (1:1,000)	Swant: CB38	
Parvalbumin	Rabbit polyclonal made from purified parvalbumin	1:1,000	Novus: NB120-11427	
Calretinin	Rabbit polyclonal to 99 aa epitope from the internal region of rat calretinin	1:1,000	Millipore: ABN2191	
TrkA	Rabbit polyclonal to extracellular domain of rat TrkA receptor	1:500	Millipore: 06-574	
$p75^{NTR}$	Mouse monoclonal to aa 1–160 from A875 melanoma cells; Clone NGFR Ab-1	1:500	NeoMarkers/Thermo Scientific MS-394-P1	Biotinylated goat anti-mouse IgG Vector Laboratories: BA9200 *Cy3 Donkey anti-mouse IgG Jackson ImmunoResearch Laboratories: 715165151
*SMI-32	Mouse monoclonal to anti-Neurofilament H, non-phosphorylated	1:2,000 (1:500)	Biolegend: 801701	
SMI-34	Mouse monoclonal to anti-Neurofilament H, phosphorylated	1:1,000	Biolegend: 835503	
*Parvalbumin	Mouse monoclonal produced with purified parvalbumin	(1:50)	Swant: PV235	*Cy3 Donkey anti-mouse IgG Jackson ImmunoResearch Laboratories: 715165151

**Fluorescent primary and secondary antibodies; IH, immunohistochemistry; IF, immunofluorescence.*

Quantitative Morphometric and Densitometric Measurements

Purkinje cells were quantified in H&E and cresyl violet stained sections in 10 randomly selected fields in a section using a 20× objective with an area of 0.20 mm^2 per field and presented as mean counts. Thickness of the cerebellar granular cell layer (GL) and molecular layer (ML) was quantified in 10 randomly chosen fields from cresyl violet stained sections using the same parameters as described above. Calb-, Parv-, SMI-32-, TrkA-, and $p75^{NTR}$-ir PC, ML Parv-ir interneuron, and Calr-ir Golgi, Lugaro, and unipolar brush cell counts in the GL were performed in two sections, 10 fields per section at 20× magnification, and calculated as mean per section. Non-phosphorylated SMI-32- and phosphorylated SMI-34-immunolabeled fusiform PC axonal swellings (i.e., torpedoes) in the GL, ML, and PC layers were quantified in one entire section per case at 10× magnification and reported as mean number of torpedoes per total cerebellar area (cm^2). Optical density (OD) measurements of Calb, Parv, SMI-32, $p75^{NTR}$, and TrkA PC neurons were measured in 10 fields per section within two sections at 40× magnification covering an area of 0.40 mm^2/per field and calculated as means per section. Additional OD measurements of Calb- and $p75^{NTR}$-ir ML dendritic arborization were quantified in 10 fields per section at 40× magnification as described above. Background measurements were averaged and subtracted from the mean OD values for each immunostained section. Quantitation and photography were performed with the aid of a Nikon Eclipse microscope coupled with NIS-Elements imaging software (Nikon, Japan).

APP/Aβ (6E10), and $Aβ_{42}$-ir plaque load were evaluated in two sections within ten randomly selected fields per section at 20× magnification covering an area of 0.20 mm^2/per field within the ML. Plaque load was calculated as percent area per cerebellar field and presented as mean number per section.

Statistical Analysis

Analysis of cell counts, OD measurements, and demographic differences between HC, DS, and AD cases were evaluated using Chi-squared (comparing gender), non-parametric Mann–Whitney rank test, and Kruskal–Wallis ANOVA on ranks test, followed by Dunn's *post-hoc* test for multiple comparisons. Correlations between variables and demographics were performed using a Spearman's test. Data significance was set at $p < 0.05$ (two-tailed). A sub-analysis was performed excluding the DSD– subjects from the DS group. Group differences for each marker were adjusted for age and gender using an analysis of covariance (ANCOVA) with and without DSD– cases after log-transforming variables that did not meet the assumption of normality. For both ANCOVAs, false discovery rate (FDR) was used to correct for multiple comparisons and α was set at < 0.01.

RESULTS

Case Demographics

Average age for each group was 70.90 (±12.60) for HC, 81.70 (±8.00) for AD, and 51 (±6.4) years for DS cases. Statistical analysis revealed a significantly lower age for the DS ($p < 0.05$) compared to both the HC and AD subjects (**Table 2**). There was no significant difference in gender frequency between groups (Chi-squared, $p = 0.9$). Average brain weight was significantly lower for DS (954.50 ± 121.90 g) than the HC (1259.40 ± 70.73 g) (Kruskal–Wallis, $p = 0.002$) but not compared to AD (1108.50 ± 177.00 g) (**Table 2**). A significantly higher post-mortem interval was found for the HC (PMI, 13.88 ± 5.40 h) compared to AD, (5.20 ± 1.19 h) (Kruskal–Wallis, $p < 0.05$) but not DS (7.48 ± 5.58 h) (**Table 2**). Braak NFT stages were significantly higher in DS and AD compared to HC cases (Kruskal–Wallis, $p \leq 0.001$) (**Table 2**). DSD+ was Braak VI compared to Braak V for the DSD– cases. For AD, 77% were Braak VI and 23% Braak V. Within the HC group 50% had a Braak score of I–III, while the others were stage IV–V. These latter cases may have brain reserve allowing for the clinical diagnosis of no dementia (Mufson et al., 2016).

Cerebellar Amyloid and Tau Pathology

The presence of Aβ plaques and NFTs in the cerebellar cortex was determined using antibodies against APP/Aβ (6E10), $A\beta_{40}$, $A\beta_{42}$, and AT8, an antibody that marks tau phosphorylation. Although APP/Aβ-ir deposits were found in each HC, DS, and AD case, only 12.5% of HC and 70% of AD cases displayed $A\beta_{42}$-ir plaques. On the other hand, $A\beta_{42}$-ir plaques were observed in both DSD+ and DSD– cases. Qualitative evaluation revealed diffuse aggregates of APP/Aβ-ir plaques scattered within the ML in both DS and AD (**Figures 1B,C**) compared to small rounded APP/Aβ-ir deposits in the GL and PC layers in both groups, while very few plaques were found in HC cases (**Figure 1A**). $A\beta_{42}$-ir plaques were observed mainly in the ML (**Figures 1E,F**) in both DS and AD but were not observed in HC (**Figure 1D**). Diffuse $A\beta_{42}$- and APP/Aβ-ir plaques within the ML displayed amyloid positive filaments (**Figures 1G,H**). In addition, APP/Aβ and $A\beta_{42}$-ir leptomeningeal arteries, arterioles, and/or capillaries (**Figure 1I**) were observed in 75% of DS, 30% of AD, and 12.5% of HC cases. $A\beta_{40}$ immunoreactivity was observed in leptomeningeal arteries in 66% of DS, 30% of AD, and 12.5% of HC cases. Interestingly, PCs were $A\beta_{42}$ and APP/Aβ immunonegative and neither $A\beta_{40}$ plaques nor AT8-ir profiles were observed in the cerebellar cortex across groups.

Quantitation of Amyloid Plaque Load

Amyloid plaque load was measured within the ML using APP/Aβ (6E10) and $A\beta_{42}$ antibodies. $A\beta_{42}$-ir plaque load was significantly higher than APP/Aβ in DS with or without dementia (Mann–Whitney, $p \leq 0.001$), while no differences were detected between $A\beta_{42}$ and APP/Aβ load in AD (data not shown). HCs displayed a higher APP/Aβ than $A\beta_{42}$ load (Mann–Whitney, $p < 0.01$, data not shown). APP/Aβ- (**Figure 2A**) and $A\beta_{42}$-ir (**Figure 2B**) plaque loads were significantly increased in DS compared to HC cases (Kruskal–Wallis, $p < 0.01$), while DS $A\beta_{42}$-ir plaque load was greater than AD (Kruskal–Wallis, $p = 0.01$). Similar results were observed when DSD– cases were removed from the statistical analysis for both antibodies. Adjusting for age and gender revealed a significantly greater $A\beta_{42}$ plaque load in DS compared to HC (ANCOVA, $p = 0.001$) and AD (ANCOVA, $p = 0.001$) (**Figure 2C**) but no difference in APP/Aβ plaque load (ANCOVA, $p > 0.01$) (**Figure 2D**) between groups. A sub-analysis removing DSD– cases showed a significantly greater $A\beta_{42}$ (ANCOVA, $p < 0.001$) and APP/Aβ plaque (ANCOVA, $p = 0.03$) load in DSD+ compared to HC. Furthermore, the ratio between $A\beta_{42}$ and APP/Aβ-ir plaque load was greater in DS compared to HC (Kruskal–Wallis, $p < 0.001$) (**Figure 2E**) and AD (Kruskal–Wallis, $p < 0.04$) (**Figure 2E**). Eliminating DSD– cases from the analysis resulted in a significantly higher $A\beta_{42}$:APP/Aβ-ir plaque load ratio in DSD+ than HC cases (Kruskal–Wallis, $p < 0.001$) but not in AD (Kruskal–Wallis, $p < 0.001$). Adjusting for age and gender, with and without DSD–, revealed no differences in $A\beta_{42}$:APP/Aβ-ir plaque load ratio between groups (ANCOVA, $p > 0.01$) (**Figure 2F**).

Quantitation of H and E and Cresyl Violet Stained PC Cells

H&E and cresyl violet stained sections were used to count PCs. GL and ML thickness were evaluated using cresyl violet due to greater laminar differentiation. In all groups, H&E and cresyl violet stained PCs but not dendrites (**Figures 3A–C**). Surprisingly, quantitation revealed fewer cresyl violet compared to H&E positive PCs in DS and AD compared to HC (Mann-Whitney, $p < 0.01$, data not shown). There was also a significant reduction in number of H&E and cresyl violet stained PCs in AD compared to HC cases (Kruskal–Wallis, $p < 0.05$) (**Figures 4A,B**), but not DS. Furthermore, there were no significant differences in GL and ML laminar thickness between groups (Kruskal–Wallis, $p > 0.05$, data not shown). A sub-analysis removing DSD– cases showed similar findings. Adjusting for age and gender revealed no significant differences in the number of H&E and cresyl violet stained PCs (**Figures 4D,E**) nor GL and ML thickness between groups, even when DSD– cases were removed from the evaluation. In addition, no significant differences were found in the number of cresyl violet compared to H&E-stained PCs between DS groups (Kruskal–Wallis, $p > 0.05$) (**Supplementary Figure 2A**), even after adjusting for age and gender (ANCOVA, $p > 0.01$; **Supplementary Figure 2B**).

SMI-32 and SMI-34 Neurofilament PC Counts

The number of PCs was also examined using antibodies against SMI-32, a somato-dendritic neuronal marker that preferentially labels non-phosphorylated neurofilaments in soma and dendrites. SMI-34 recognizes phosphorylated intermediate neurofilaments of high-molecular-weight. SMI-32 immunostaining was observed in PC soma, dendrites, and axons (**Figures 3D–F**) in all groups. SMI-34 immunostained basket cell (**Figures 5B,G**) axons, PC axons (**Figures 5E,F**), and parallel fibers across cohorts (**Figures 5A,C,D,F–I**). Although PC soma

TABLE 2 | Case demographics.

	HC ($n = 8$)	DS ($n = 12$)*	AD ($n = 10$)	p-value	Group-wise comparisons (p-value)
Age (years)	70.88 ± 12.55 [51–85][a]	51.00 ± 6.41 [44–60]	81.70 ± 7.97 [71–98]	<0.001[b]	DS < HC (0.011) DS < AD (<0.001)
Male/Female (n)	3/5	4/8	3/7	0.9[c]	–
PMI (h)	13.88 ± 5.41 [3–20][a]	7.48 ± 5.58 [2.2–20]	5.20 ± 1.18 [3.5–6]	0.029[b]	AD < HC (0.037)
Brain weight (g)**	1259.40 ± 70.73 [1180–1400][a]	954.40 ± 121.92 [700–1090]	1108.50 ± 177.00 [925–1430]	0.003[b]	DS < HC (0.002)
Braak scores (n)***	I [1], II [2], III [1], IV [3], V [1]	V [3], VI [9]	V [2], VI [7]	≤0.001[b]	HC < AD, DS (≤0.001)

Dementia DS n = 9; non-dementia DS n = 3.
***1 dementia DS subject and 1 non-dementia DS subject do not have brain weight data.*
****1 AD subject does not have a Braak score.*
[a]*Mean ± SD [range].*
[b]*Kruskal–Wallis test.*
[c]*Chi-squared.*

were SMI-34 immunonegative (**Figures 5B,G**), we observed an increase in SMI-34 immunopositive parallel fibers within the superficial portion of the ML in the oldest cases independent of group (**Figures 5A,C,D**).

Quantitative analysis revealed a significant decrease in the number of SMI-32-ir PCs in AD compared to HC (Kruskal–Wallis, $p = 0.001$) (**Figure 4C**) but not in DS. A sub-analysis removing DSD– cases found a significant reduction in SMI-32-ir PCs in DSD+ compared to HC cases (Kruskal–Wallis, $p = 0.02$). SMI-32-ir PC ODs were significantly higher in DS compared to HC ($p = 0.029$) but not AD subjects. This significant difference was lost when the DSD– cases were eliminated from the analysis ($p = 0.09$). Adjusting for age and gender, HC subjects displayed a significantly higher number of positive SMI-32 PCs than AD or DS with or without dementia (ANCOVA, $p < 0.001$) (**Figure 4F**), with no difference in SMI-32-ir PC OD intensity among groups (ANCOVA, $p > 0.01$) with or without dementia DS cases. Furthermore, the ratio of the number of SMI-32-ir PCs to H&E PC numbers in AD and DS with (Kruskal–Wallis, $p = 0.03$) or without dementia (Kruskal–Wallis, $p = 0.04$) was lower compared to HC cases (**Supplementary Figure 2C**). Adjusting for age and gender yielded a similar result only when DSD– cases remained in the analysis (ANCOVA, $p = 0.01$, **Supplementary Figure 2D**).

SMI-32 and SMI-34 immunostaining also revealed swellings mainly in proximal PC axons termed torpedoes/spheroids (Bouman, 1918) (**Figures 3G–I**, **5E,F**). While SMI-32-ir PC torpedoes were found primarily in the GL in 60% of AD, 33% of DS, and 25% of HC cases, SMI-34-ir torpedoes were observed in all three cerebellar layers in each group. Although SMI-34-ir torpedoes were less abundant in both ML and PC layers compared to the GL, a few were seen in PC dendrites (**Figure 5I**). Counts of SMI-34-ir torpedoes in the GL revealed significantly higher numbers in both AD and HC cases compared to DS (Kruskal–Wallis, $p < 0.001$) (**Figure 6B**), even when DSD– cases were removed from the analysis (Kruskal–Wallis, $p < 0.001$), while SMI-32 torpedo numbers were similar between groups (Kruskal–Wallis, $p > 0.05$) (**Figure 6A**). Controlling for age and gender yielded similar results for SMI-32 (**Figure 6C**), while the number of SMI-34-ir torpedoes were significantly higher in AD compared to DS independent of dementia, but not HC (ANCOVA, $p = 0.01$) cases (**Figure 6D**). Furthermore, the number of SMI-34-ir torpedoes were significantly higher than the number of SMI-32 torpedoes in all three groups (Mann–Whitney, HC $p < 0.001$, DS $p = 0.024$, and AD $p = 0.004$, data not shown).

Calbindin, Parvalbumin, and Calretinin Cell Counts

Calbindin immunostaining was only seen in PCs (**Figures 7A–C**, **Supplementary Figures 3A,D,G**), whereas Parv immunoreactivity was also observed in cells within the ML, most likely stellate and basket interneurons, in each group (**Figures 7D–F**, **Supplementary Figures 3B,E**). Small Parv-ir interneurons were less evident in DS (**Figure 7E**) compared to HC (**Figure 7D**) and AD (**Figure 7F**) cases (see also **Supplementary Figures 3A–F**). Both CBPs were detected in PC soma, proximal and distal dendrites, and axons in HC, DS, and AD cases. However, PC proximal dendrites and axons were less immunoreactive for these CBPs in DS (**Figures 7B,E**) compared to HC (**Figures 7A,D**) and AD (**Figures 7C,F**). Calb-ir axonal torpedoes were observed in HC, DS, and AD subjects (**Figures 7A1–C1**), while positive dendrites were rare (**Supplementary Figures 3G,I**). Quantitation revealed no differences in Calb-ir PC number, PC soma and dendritic arborization OD values between groups, with or without DSD– cases (Kruskal–Wallis, $p > 0.05$) (**Figure 8A**). In contrast, Parv-ir PC numbers were significantly reduced in AD compared to HC ($p = 0.001$) (**Figure 8B**), while OD values for Parv-ir PC soma were significantly greater in DS than AD ($p < 0.001$). No differences in Parv-ir PC counts or ODs were detected in DS compared to HC cases. However, the number of ML Parv-ir interneurons was significantly reduced in DS compared to HC (Kruskal–Wallis, $p < 0.001$) and AD (Kruskal–Wallis, $p < 0.001$) (**Figure 8C**). Removal of DSD– cases did not alter these findings. Adjusting for age and gender revealed no statistical differences in number of Calb-ir PC and OD dendritic arborization values among groups, with or without inclusion of DSD– cases (**Figure 8D**). Counts of Parv-ir PCs revealed a significant reduction in DS and AD compared to the HC group (ANCOVA,

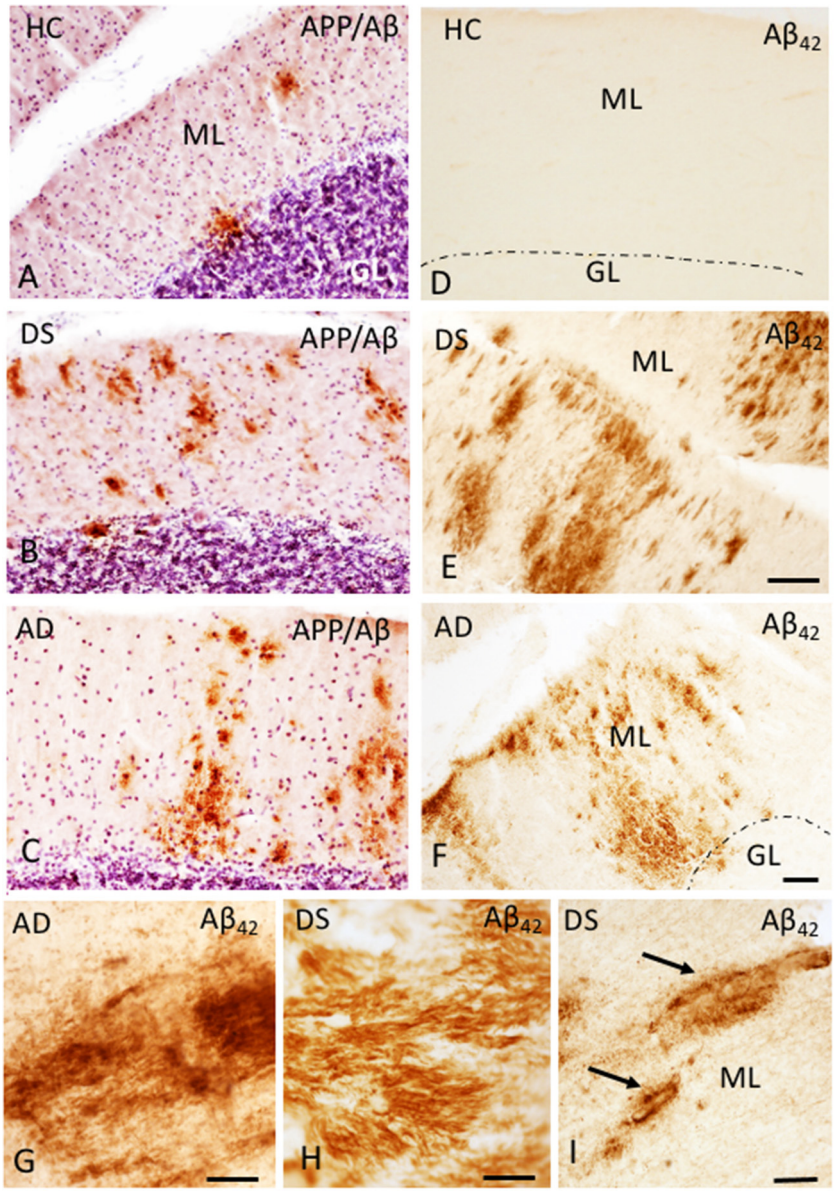

FIGURE 1 | Photomicrographs showing a few scattered APP/Aβ- **(A)** and the absence of $A\beta_{42}$-ir **(D)** plaques in the ML of the cerebellar cortex of a 66-year-old female HC **(A,D)** compared to numerous APP/Aβ- **(B,C)** and $A\beta_{42}$-ir **(E,F)** plaques in a 46-year-old male with DSD+ **(B,E)** and a 98-year-old female with AD **(C,F)** case, respectively. High-power photomicrographs of $A\beta_{42}$-ir filament-like bundles within diffuse plaques in a 98-year-old female with AD **(G)** and 47-year-old female with DSD– **(H)**, and $A\beta_{42}$-ir blood vessels (arrows) in a 46-year-old male with DSD+ **(I)**. **(A–C)** were counterstained with Gill's hematoxylin to aid in the visualization of cerebellar laminae. HC, healthy control; DS, Down syndrome; AD, Alzheimer's disease; ML, molecular layer; GL, granular cell layer. Scale bars: **(E)** = 100 μm applies to **(A,B,D)**; **F** = 100 μm applies to **(C)**; **G,H** = 10 μm; **(I)** = 50 μm.

$p < 0.001$) independent of DS clinical status (**Figure 8E**). There were no statistical differences in Parv-ir PC OD values among groups with and without DSD– cases. Parv-ir interneurons were reduced in DS with or without dementia compared to HC and AD cases (**Figure 8F**) (ANCOVA, $p < 0.001$).

Calretinin profiles were found in the GL and ML in all cases examined (**Figures 9A–L**). Calr-ir neurons in the GL displayed features indicative of Lugaro (**Figure 9G**), unipolar brush (**Figures 9H,I,K,L**), and Golgi (**Figure 9J**) interneurons (Diño et al., 1999; Stepień et al., 2012). Calr-ir beaded fibers were seen in close proximity to PC dendrites (**Figures 9B,D,F**) and forming rosettes in the GL (**Figures 9G–I**), likely corresponding to cerebellar climbing and mossy fibers (Rogers, 1989; Álvarez et al., 2008), respectively, in all three groups (**Figures 9B,D,F,G–I**). Brush cells showed stronger Calr immunoreactivity compared to Lugaro and Golgi interneurons (**Figures 9A,C,E,G–L**). In contrast to HC and AD subjects, Calr-ir cell types were less numerous in DS (**Figures 9A,C,E**). Counts revealed a significant

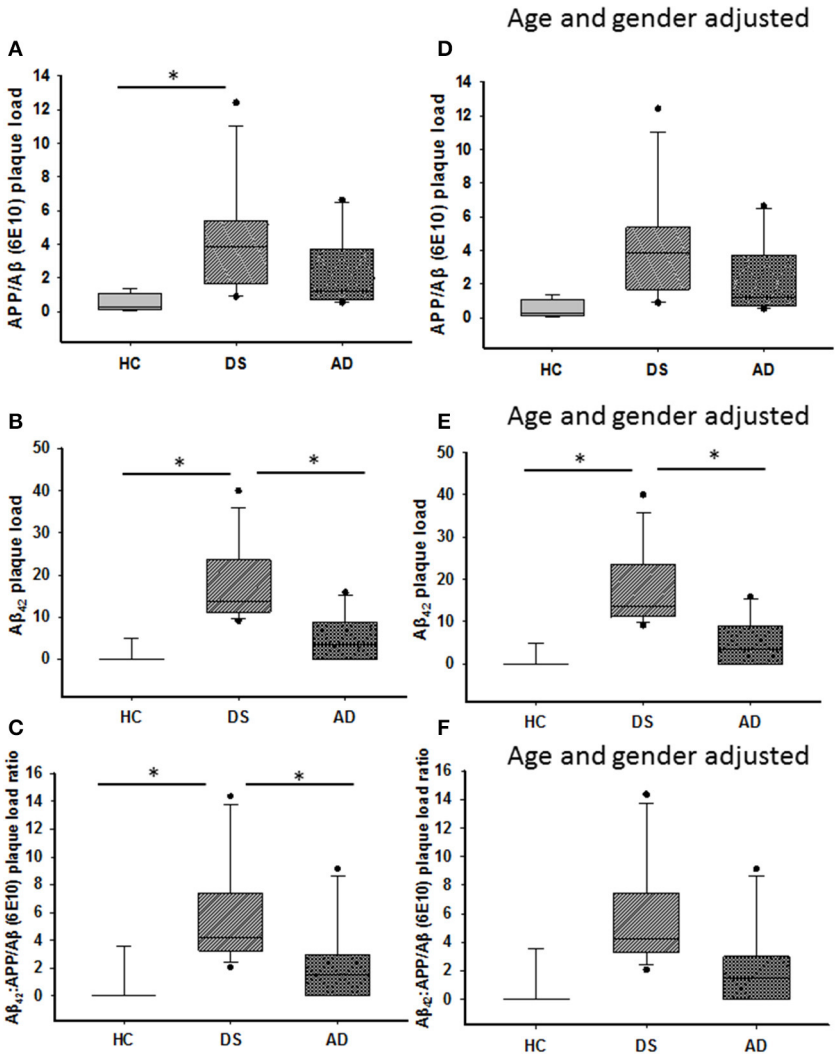

FIGURE 2 | Box plots showing significantly higher APP/Aβ- **(A)** and $A\beta_{42}$-ir **(B)** plaque loads, as well as $A\beta_{42}$:APP/Aβ **(C)** plaque load ratio in the ML of DS compared to HC cases (Kruskal–Wallis, $p < 0.01$), while $A\beta_{42}$-ir plaque load (**B**; Kruskal–Wallis, $p = 0.01$) and $A\beta_{42}$:APP/Aβ plaque load ratio (**C**; Kruskal–Wallis, $p = 0.04$) were significantly greater in DS compared to AD. Adjusting for age and gender revealed a greater ML $A\beta_{42}$ plaque load in DS compared to HC and AD (**E**; ANCOVA, $p = 0.001$), with no difference in APP/Aβ **(D)** plaque load and $A\beta_{42}$:APP/Aβ **(F)** ratio between groups (ANCOVA, $p > 0.01$). ANCOVA, analysis of covariance. * denotes significance between groups.

reduction in the number of total GL Calr-ir cells in DS compared to HC (Kruskal–Wallis, $p < 0.001$) and AD (Kruskal–Wallis, $p < 0.002$) (**Figure 9M**). Furthermore, the numbers of Calr-ir Golgi, Lugaro, and brush cells were also significantly decreased in DS compared to HC (Golgi and brush: Kruskal–Wallis, $p < 0.001$; and Lugaro: Kruskal–Wallis, $p = 0.01$) and AD (Golgi and brush: Kruskal–Wallis, $p < 0.01$; and Lugaro: Kruskal–Wallis, $p < 0.001$) (**Figure 9O**). Similar results were obtained when DSD– cases were eliminated from the analysis. Adjusting for age and gender showed a significant decrease in both the total number of Calr-ir interneurons and Calr-ir Golgi cells in DS compared to AD and HC (ANCOVA, $p < 0.001$), while the number of Lugaro (ANCOVA, $p = 0.04$) and brush (ANCOVA, $p = 0.005$) Calr containing cells were greater in HC compared to DS with or without dementia (**Figures 9N,P**) but not AD. Calr-positive Lugaro cell numbers were not significantly different between HC and DS even when DSD– cases were removed from the analysis.

Quantitation of $p75^{NTR}$ and TrkA-Positive PCs

Purkinje cells soma displayed $p75^{NTR}$ and TrkA immunoreactivity across all the groups (**Figure 10**). Although PC $p75^{NTR}$ dendritic trees extended into ML, similar TrkA-positive profiles were not observed in HC (**Figure 10D**), DS (**Figure 10E**) or AD (**Figure 10F**). $P75^{NTR}$ but not TrkA-ir torpedoes were seen in the GL in DS, AD, and HC subjects (data not shown).

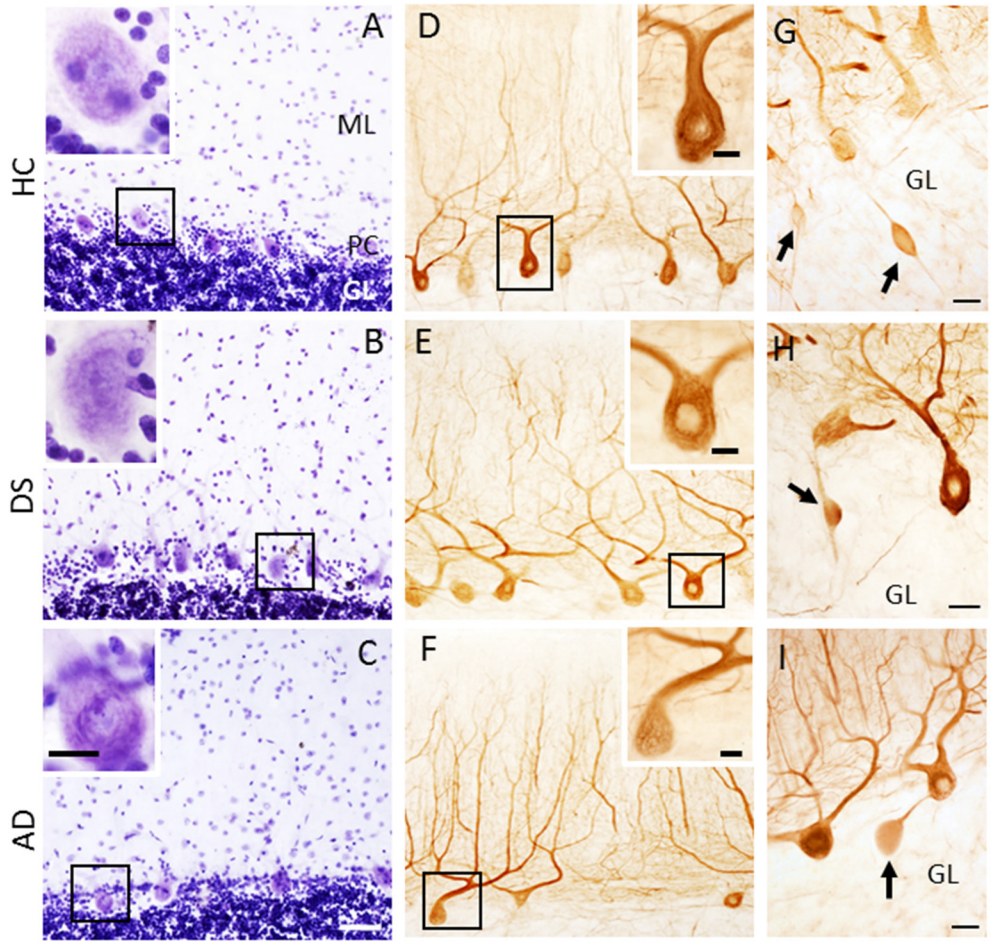

FIGURE 3 | Low-power photomicrographs of cresyl violet staining of the cerebellar cortex showing the ML, PC layer, and GL in 63-year-old male HC **(A)**, 46-year-old male with DSD+ **(B)**, and 79-year-old female with AD **(C)** cases. Insets in the upper left corners show high-power images of PC perikaryon outlined in the black boxes in **(A–C)**. Note the absence of the PC dendritic arbor **(A–C)**. Photomicrographs of SMI-32-ir non-phosphorylated high-molecular-weight neurofilaments in PC dendritic arbors and axons in a female 69-year-old HC **(D)**, male 46-year-old DSD+ **(E)** and a male 85-year-old AD **(F)** case. Insets in **(D–F)** show high-power images of boxed SMI-32-ir PCs and proximal dendrites. **(G–I)** Swollen SMI-32-ir proximal PC axons or torpedoes (arrows) in the GL of a male 51-year-old HC **(G)**, 60-year-old female with DS **(H)** and 98-year-old female with AD **(I)** case. Scale bars: **(C)** = 50 μm applies to **(A,B,D–F)**; inset in **(C)** = 15 μm, insets in **(A,B,D–F)** = 10 μm; **(G–I)** = 25 μm.

Quantitation showed higher numbers of TrkA- compared to $p75^{NTR}$-ir PCs in all the groups (Mann–Whitney, $p \leq 0.001$). Counts between groups revealed a significant reduction in the number of $p75^{NTR}$-ir PCs in both AD (Kruskal–Wallis, $p = 0.001$) and DS (Kruskal–Wallis, $p = 0.03$) compared to HC (**Figure 11A**). When DSD– cases were removed from the analysis, the significance of this reduction was increased in DS compared to HC (Kruskal–Wallis, $p = 0.004$). The OD measurement of PC soma immunoreactive for $p75^{NTR}$ was significantly higher in HC compared to subjects with AD (Kruskal–Wallis, $p = 0.009$) (**Figure 11B**) but not different from DS independent of phenotype. By contrast, OD measurement of the PC dendritic tree displaying $p75^{NTR}$ immunoreactivity revealed no significant difference between DS groups with or without dementia (Kruskal–Wallis, $p > 0.05$). The number of TrkA-positive PCs was significantly lower in AD compared to HC but not DS (Kruskal–Wallis, $p < 0.05$) (**Figure 11C**) with or without DSD– cases. TrkA-positive PC OD measurements showed a trend toward a decrease in AD and DS compared to HC subjects but did not reach significance when analyzed with (Kruskall–Wallis, $p = 0.065$) or without DSD– (Kruskall–Wallis, $p = 0.059$) cases.

Adjusting for age and gender revealed that the number of $p75^{NTR}$ and TrkA-positive PCs was significantly reduced in AD compared to HC (ANCOVA, $p < 0.011$) (**Figures 11D,F**), but no difference was found in the DS cases. In addition, $p75^{NTR}$, but not TrkA-positive PC number was significantly decreased in DS compared to HC when DSD– cases were eliminated

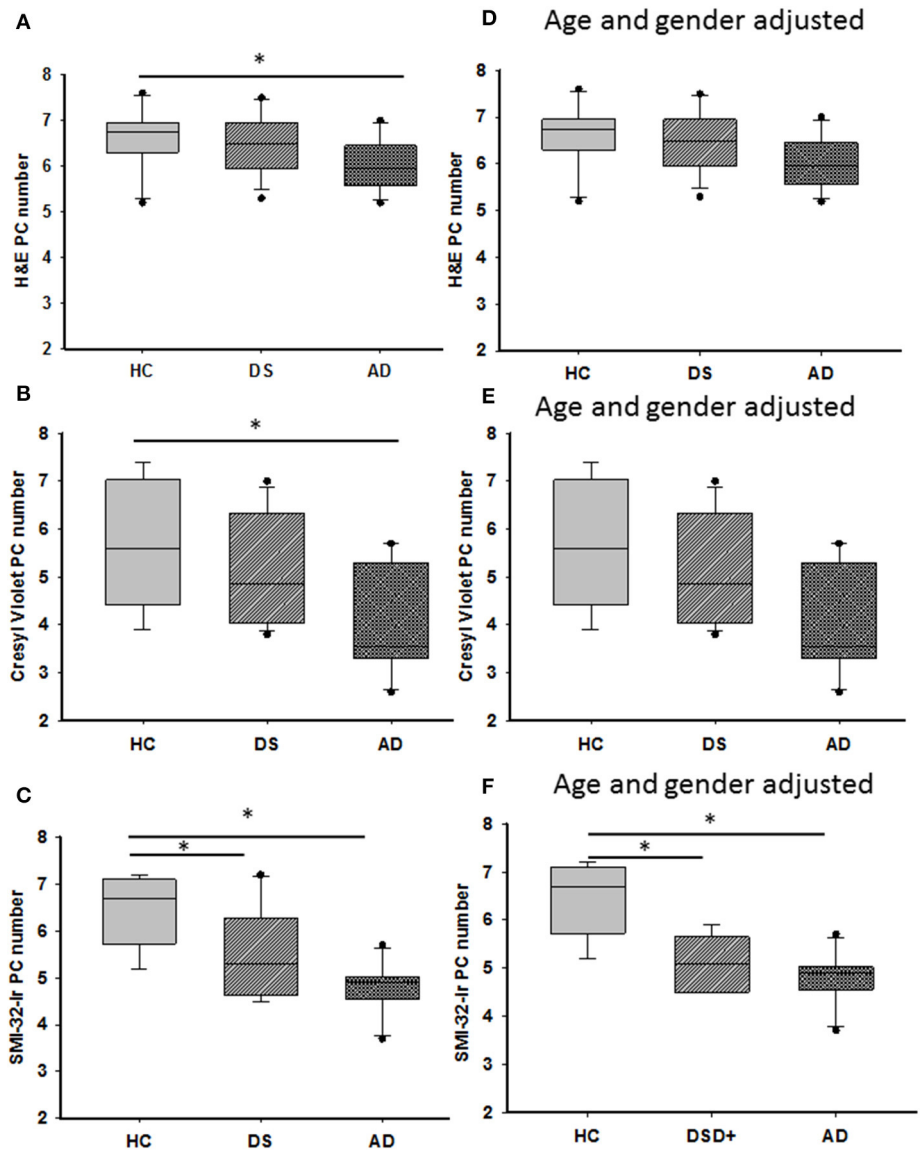

FIGURE 4 | Representative box plots showing a significant reduction in H&E **(A)** and cresyl violet **(B)** stained and SMI-32 immunoreactive **(C)** PCs in AD compared to HC cases (Kruskal–Wallis, $p < 0.05$), as well a significant reduction in the number of SMI-32-ir PCs in DS compared to HC (**C**; Kruskal–Wallis, $p = 0.001$). Adjusting for age and gender revealed that the number of H&E **(D)** and cresyl violet **(E)** stained PCs remained unchanged between groups as did counts for SMI-32 positive PCs (**F**; ANCOVA, $p < 0.001$). H&E, hematoxylin and eosin. * denotes significance between groups.

from the analysis (ANCOVA, $p < 0.001$) (**Figure 11D**). $P75^{NTR}$-ir PC perikaryon, but not dendritic arborization OD values were significantly greater in HC compared to AD (ANCOVA, $p = 0.009$) (**Figure 11E**) but not DS. A sub-analysis removing the DSD– cases revealed similar results (ANCOVA, $p = 0.004$).

Correlations Between Cerebellar Neuron Counts and Case Demographics

We found positive correlations between H&E and cresyl violet stained PC number ($r = 0.482$, $p = 0.007$) and GL and ML thickness ($r = 0.752$, $p = 0.0000002$) across the groups. A significant positive correlation was found between APP/Aβ and $A\beta_{42}$ plaque load ($r = 0.838$, $p = 0.0000002$) within the ML. Number of PC SMI-32-positive neurons correlated positively with Parv-ir (**Figure 12B**; $r = 0.69$, $p = 0.0000087$), $p75^{NTR}$-ir (**Figure 12C**; $r = 0.075$, $p = 0.0000002$), TrkA-ir (**Figure 12D**; $r = 0.67$, $p = 0.000024$), and cresyl violet stained neurons in this region, but to a lesser degree with Calb-ir PC counts (**Figure 12A**; $r = 0.47$, $p = 0.0085$) across the groups. In addition, we found a positive correlation between PC TrkA-ir number and TrkA-ir PC soma OD measurements (**Supplementary Figure 4B**; $r = 0.57$, $p = 0.0053$). However, no significant correlations were found between PC TrkA and $p75^{NTR}$ soma OD values. There was a correlation between the number of Parv and Calb PCs

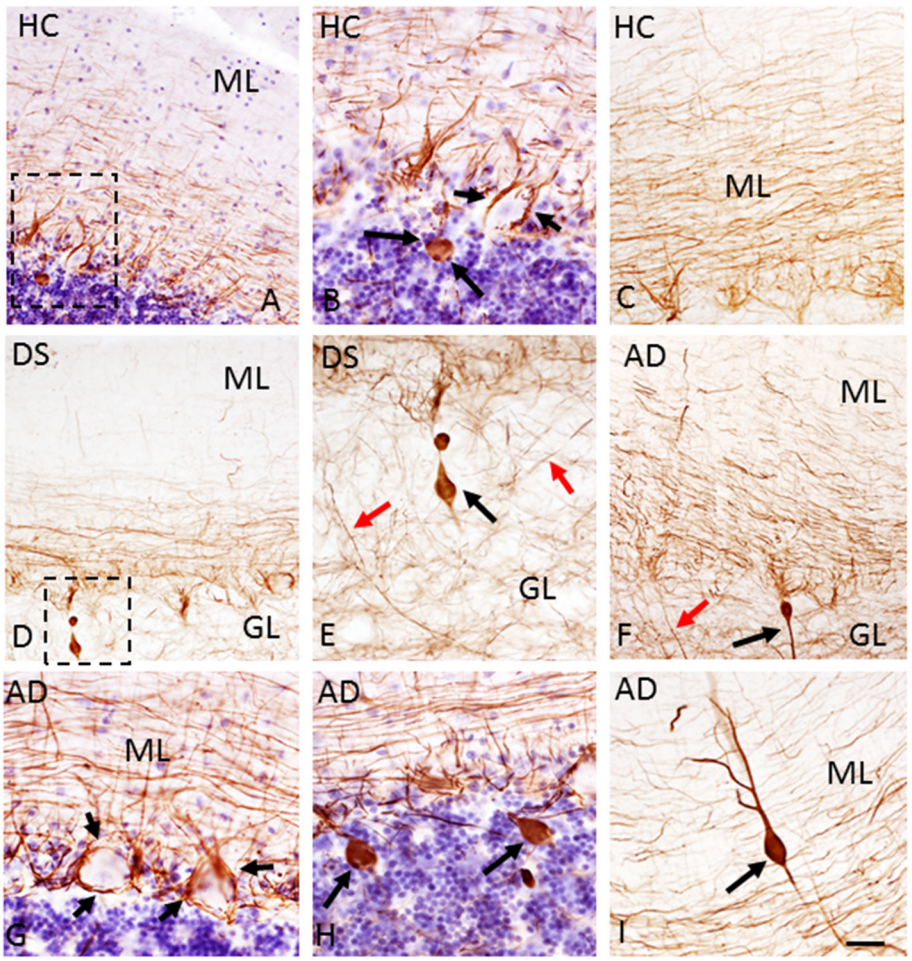

FIGURE 5 | Photomicrographs showing SMI-34-immunoreactivity in parallel fibers, basket cell axons around PCs (**B,G**; small arrows), PC axons (**E,F**; red arrows) and torpedoes (**A,B,D–F,H,I**; larger black arrows) in a 51-year-old male HC **(A,B)**, **(C)** 85-year-old male HC, a 47-year-old female with DSD– **(D,E)**, a 79-year-old female **(G,H)**, and 88-year-old male with AD **(F,I)** case. **(B,E)** Show high-power images of the cells outlined by the dotted boxes in **(A,D)**, respectively. Note the lack of SMI-34 immunostaining in PCs across groups **(A–H)** and an increase in SMI-34-ir parallel fibers in the ML of a 85-year-old male HC **(C)** compared to a 51-year-old male HC **(A)** and a 47-year-old female with DS **(D)**. Note a rare dendritic torpedo in the ML of a 78-year-old female with AD **(I)**. Tissue shown in **(A,B,G,H)** were counterstained with Gill's hematoxylin. Scale bar = 50 μm in **(A,C,D,F)** and 20 μm in **(B,E,G–I)**.

and TrkA and $p75^{NTR}$ containing PCs. Parv-ir PC numbers were not correlated with Parv PC soma OD values. SMI-32 and Parv PC soma OD values were positively correlated ($r = 0.424$, $p = 0.02$), but there was no correlation with the number of SMI-32 positive PCs. There was a positive correlation between OD values for $p75^{NTR}$ PC soma (**Figure 12F**; $r = 0.82$, $p = 0.0000002$), $p75^{NTR}$ dendritic arborization OD values (**Figure 12E**; $r = 0.55$, $p = 0.0085$), and SMI-32-ir PC counts (**Supplementary Figure 4A**; $r = 0.68$, $p = 0.00038$) across the groups. There was a strong negative correlation between the number of $p75^{NTR}$, but not TrkA-positive PCs and APP/Aβ plaque load in AD ($r = -0.923$, $p = 0.0000002$), but not in DS ($r = 0.35$, $p = 0.2$) or across groups ($r = -0.37$, $p = 0.06$). Interestingly, the numbers of GL SMI-32- and SMI-34-ir torpedoes were positively correlated between AD and HC ($r = 0.60$, $p = 0.007$) but not across all cohorts. SMI-34-ir torpedoes correlated negatively with $A\beta_{42}$ plaque load ($r = -0.60$, $p = 0.0007$) but not with APP/Aβ plaque load across the groups. Furthermore, Parv-ir stellate/basket cell counts correlated strongly with total number of Calr-ir brush, Lugaro, and Golgi interneurons (**Supplementary Figure 4C**; $r = 0.76$, $p = 0.0000002$). Golgi cell numbers correlated with Calr positive brush (**Supplementary Figure 4E**; $r = 0.73$, $p = 0.0000002$), Lugaro (**Supplementary Figure 4F**; $r = 0.68$, $p = 0.00003$), and Parv-positive interneuron counts (**Supplementary Figure 4D**; $r = 0.74$, $p = 0.0000002$) across the groups. Parv-ir interneuron counts showed a negative association with OD values for SMI-32 PC soma ($r = -0.57$, $p < 0.001$), Parv-ir neurons ($r = -0.48$, $p = 0.008$), $A\beta_{42}$ plaque load (**Supplementary Figure 5A**; $r = -0.74$, $p = 0.0000002$), and APP/Aβ (**Supplementary Figure 5B**; $r = -0.61$, $p = 0.00033$) plaque load. We also found negative correlations between the number of GL Calr positive cells, $A\beta_{42}$ plaque load

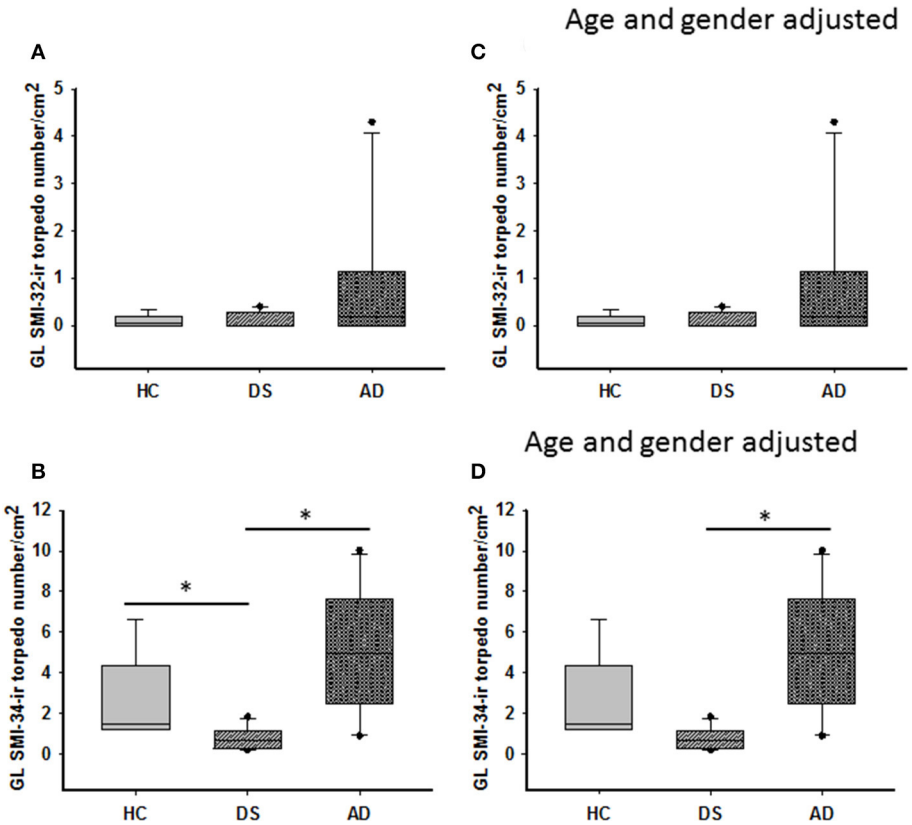

FIGURE 6 | Box plots showing no difference in the number of GL SMI-32 stained axonal torpedoes between groups **(A)** even after adjusting for age and gender **(C)**. A significant decrease in GL SMI-34-ir torpedo numbers was observed in DS compared to HC and AD cases (**B**; Kruskal–Wallis, $p < 0.001$). When adjusted for age and gender, GL SMI-34-ir axonal torpedo counts were significantly reduced in DS compared to AD, but not HC (**D**; ANCOVA, $p < 0.001$). * denotes significance between groups.

(**Supplementary Figure 5C**; $r = -0.70$, $p = 0.0000002$), and APP/Aβ plaque load (**Supplementary Figure 5D**; $r = -0.47$, $p = 0.0082$) across the groups. Calr-ir Lugaro cell numbers correlated negatively with Parv-ir PC OD values ($r = -0.60$, $p < 0.001$). Number of Calr-ir brush cells correlated positively with the number of $p75^{NTR}$ PCs ($r = 0.50$, $p < 0.008$) across groups. In addition, we found strong negative correlations between Calr-ir Golgi and brush cell counts ($r = -0.65$, $p = 0.00013$) with $A\beta_{42}$ plaque load (**Supplementary Figure 5E**; $r = -0.71$, $p = 0.0000002$) but a weaker association with APP/Aβ plaque load (**Supplementary Figure 5F**; $r < -0.5$ and $p = 0.014$), while Calr-ir Lugaro counts correlated only with $A\beta_{42}$ plaque load ($r = -0.52$, $p = 0.003$).

$A\beta_{42}$ plaque load correlated negatively with age ($r = -0.630$, $p = 0.0002$). Calb ($r = -0.429$, $p = 0.018$) and Parv ($r = -0.397$, $p = 0.03$) positive PC counts displayed a weak negative association with age across groups. OD values for Parv positive PCs showed a strong negative association with increased age ($r = -0.705$, $p = 0.000002$). These age-related negative correlations are most likely due to the significantly younger age of the DS compared to the AD and HC subjects. GL SMI 34-ir, but not SMI-32 torpedo counts positively correlated with age (**Supplementary Figure 6A**; $r = 0.62$, $p = 0.0003$). Number of SMI-32 (**Supplementary Figure 6B**; $r = -0.66$ $p = 0.00007$), $p75^{NTR}$ (**Supplementary Figure 6C**; $r = -0.73$, $p = 0.0000002$), Parv (**Supplementary Figure 6D**; $r = -0.59$, $p = 0.00064$), and TrkA (**Supplementary Figure 6E**; $r = -0.57$, $p = 0.001$)-positive PC counts, but not Calb cells, showed a significant negative association with Braak NFT scores across groups. There was a negative correlation between Braak scores and both $p75^{NTR}$ (**Supplementary Figure 6F**; $r = -0.57$, $p = 0.006$) and TrkA (**Supplementary Figure 7A**; $r = -0.56$, $p = 0.008$) PC soma OD values as well as Calr positive cell counts (**Supplementary Figure 7B**; $r = -0.58$, $p = 0.00094$).

DISCUSSION

Down syndrome is characterized, in part, by cognitive impairment, which is present to some degree in all people with this disorder (Pennington et al., 2003). By age 40, all individuals with DS exhibit NFT and amyloid lesions similar to those observed in the AD brain with a concentration in neo and limbic cortical regions (Leverenz and Raskind, 1998; Head et al., 2012; Davidson et al., 2018; Perez et al., 2019), and display impairments in learning, memory, language, and motor behaviors (Rajmohan and Mohandas, 2007; Rolls,

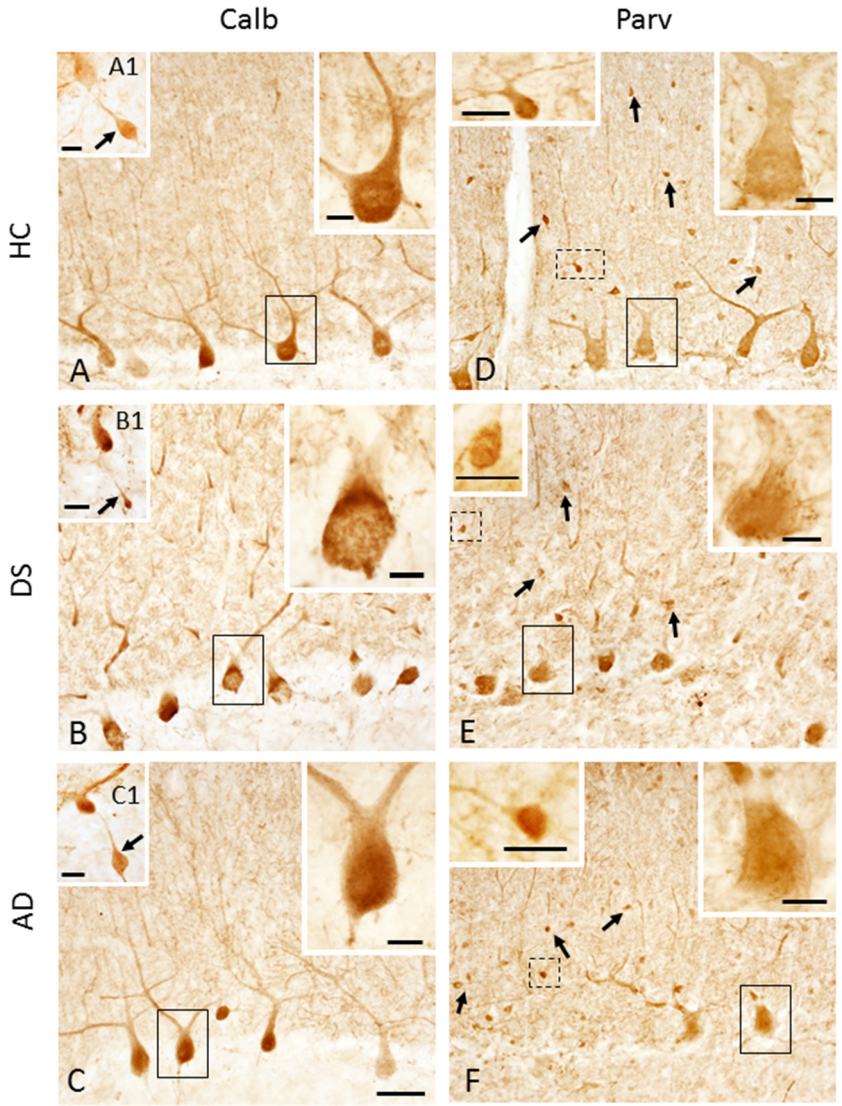

FIGURE 7 | Photomicrographs showing Calb-ir PCs in a 66-year-old female HC **(A)**, 47-year-old female with DSD– **(B)** and a 72-year-old female with AD **(C)** case. Upper right insets show high-power image of the black outlined Calb-ir PCs shown in **(A–C)**. Insets **(A1–C1)** show GL Calb-ir axonal torpedoes (arrows) in a 51-year-old male HC **(A1)**, 60-year-old female with DSD– **(B1)** and a 79-year-old female with AD **(C1)** case. **(D–F)** Parv-ir PCs and Parv-ir interneurons (black arrows) within the ML in a 69-year-old female HC **(D)**, 44-year-old female with DSD– **(E)**, and a 72-year-old female with AD **(F)**. Upper right insets **(D–F)** are higher magnification images of the Parv-ir PCs outlined in solid black boxes, while upper left insets are higher magnification photos of the Parv-ir interneurons (outlined by dashed lines) within the ML, most likely stellate and basket interneurons in **(D–F)**, respectively. Note the presence of many more Parv-ir interneurons in HC **(D)** compared to DS **(E)** and AD **(F)**. Scale bars: **(C)** = 50 μm and applies to **(A,B,D–F)**; **(A1–C1)** insets = 30 μm; larger insets in **(A–F)** = 10 μm; smaller insets in **(D–F)** = 20 μm.

2015). Neuropathological examination has shown a significant reduction in cerebellar volume/size in infants, children, and adults with DS (Guidi et al., 2011; Mufson et al., 2020) that is recapitulated in genetic mouse models of this disorder (Necchi et al., 2008; Lomoio et al., 2009). Although the cerebellum plays a key role in the regulation of proprioceptive-motor control and motor learning (Spanò et al., 1999; Malak et al., 2015), evidence suggests that it is also involved in higher order functions including cognition (Schmahmann, 2004), dysmetria of thought (Schmahmann, 1991), and the cerebellar cognitive affective syndrome (Tavano et al., 2007). Cerebellar lesions can lead to the development of a behavioral pattern characterized by reduced cognitive efficiency associated with executive and visuospatial, expressive language, and affective disorders (Tavano et al., 2007; Yildiz et al., 2010). Despite data linking cerebellar dysfunction to defects in cognition, there are virtually no detailed investigations of the cellular pathobiology of the adult cerebellum in individuals with DS. Here we present, a first of its kind report describing alterations in PCs and interneurons that contain either CBPs or the cognate NGF receptors, TrkA, and $p75^{NTR}$ and their relation

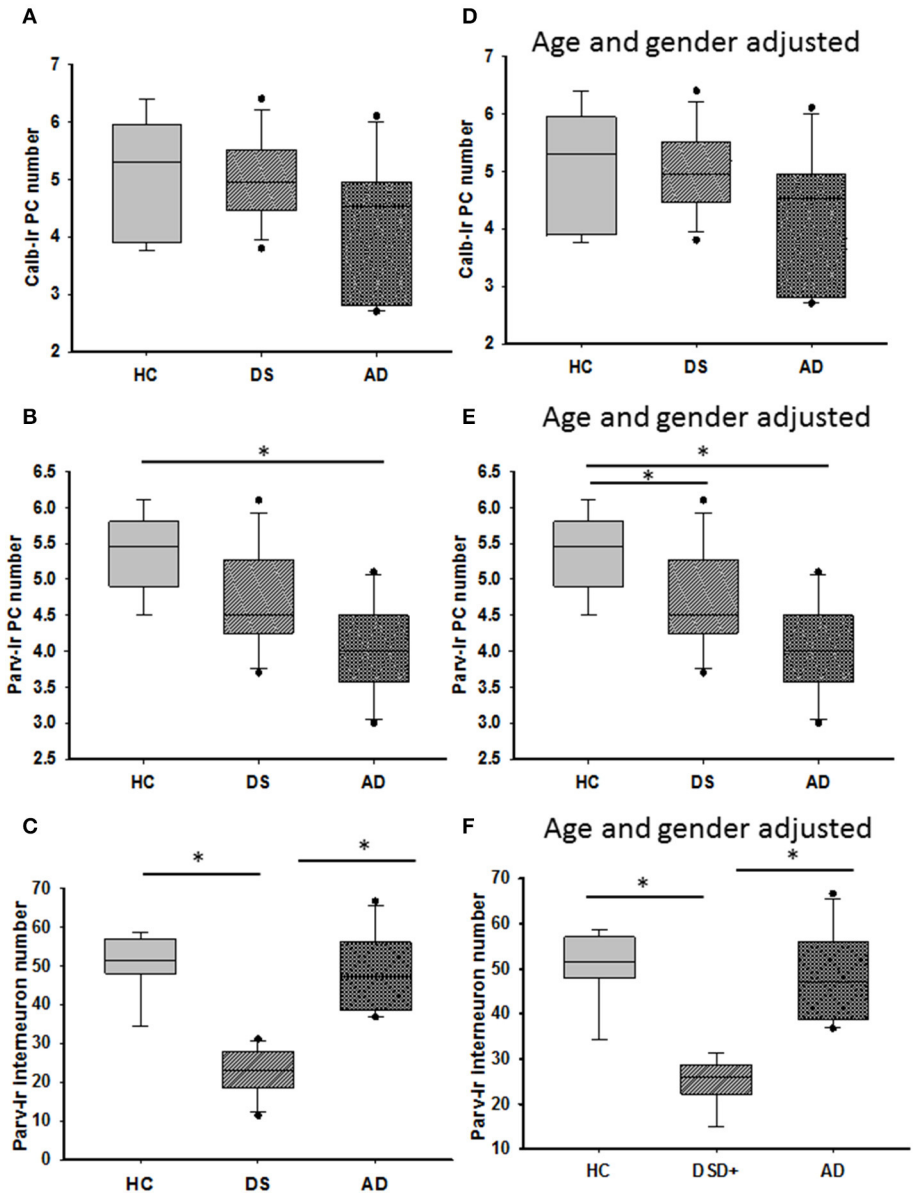

FIGURE 8 | Box plots showing no differences in the number of Calb-ir PCs **(A)** even when adjusted for age and gender **(D)** between the groups. By contrast, HCs had a significantly greater number of Parv-ir PCs compared to subjects with AD (**B**; Kruskal–Wallis, $p = 0.001$). When adjusted for age and gender, HCs had significantly more Parv positive PCs than both subjects with DS and AD (**E**; ANCOVA, $p < 0.001$), while Parv-ir interneurons showed significantly lower numbers in DS compared to HC and AD cases (**C**; Kruskal–Wallis, $p < 0.001$) even after adjusting for age and gender (**F**; ANCOVA, $p < 0.001$). * denotes significance between groups.

to AD-like pathology in the cerebellum of older people with DS compared to AD and HC.

Amyloid plaques and NFTs are common pathological manifestations that appear in most adults with DS before the age of 50 (Mann and Esiri, 1989). In the present study, we observed APP/Aβ and $A\beta_{42}$ positive cerebellar plaques and blood vessels as well as $A\beta_{40}$ leptomeningeal vessels, but not $A\beta_{40}$ plaques, or NFTs in dementia and non-dementia DS cases (Tamaoka et al., 1995; Mann et al., 1996; Wang et al., 2002). Cerebral angiopathy was more frequently seen in the cerebellar cortex in DS than AD suggesting a more compromised vasculature in DS. Within cerebellar cortex, diffuse APP/Aβ and $A\beta_{42}$ deposits were mainly found in the ML, with few scattered APP/Aβ deposits in the GL, PC layer, and white matter in both DS (Cole et al., 1993; Li et al., 1994; Cataldo et al., 1996; Mann et al., 1996) and AD (Pro et al., 1980; Joachim et al., 1989; Ogomori et al., 1989; Lemere et al., 1996; Wang et al., 2002; Mavroudis et al., 2010; Sepulveda-Falla et al., 2014; Catafu et al., 2016; Jacobs et al., 2018). $A\beta_{42}$ and APP/Aβ plaque loads were significantly greater in DS compared to HC, while only $A\beta_{42}$ plaque load

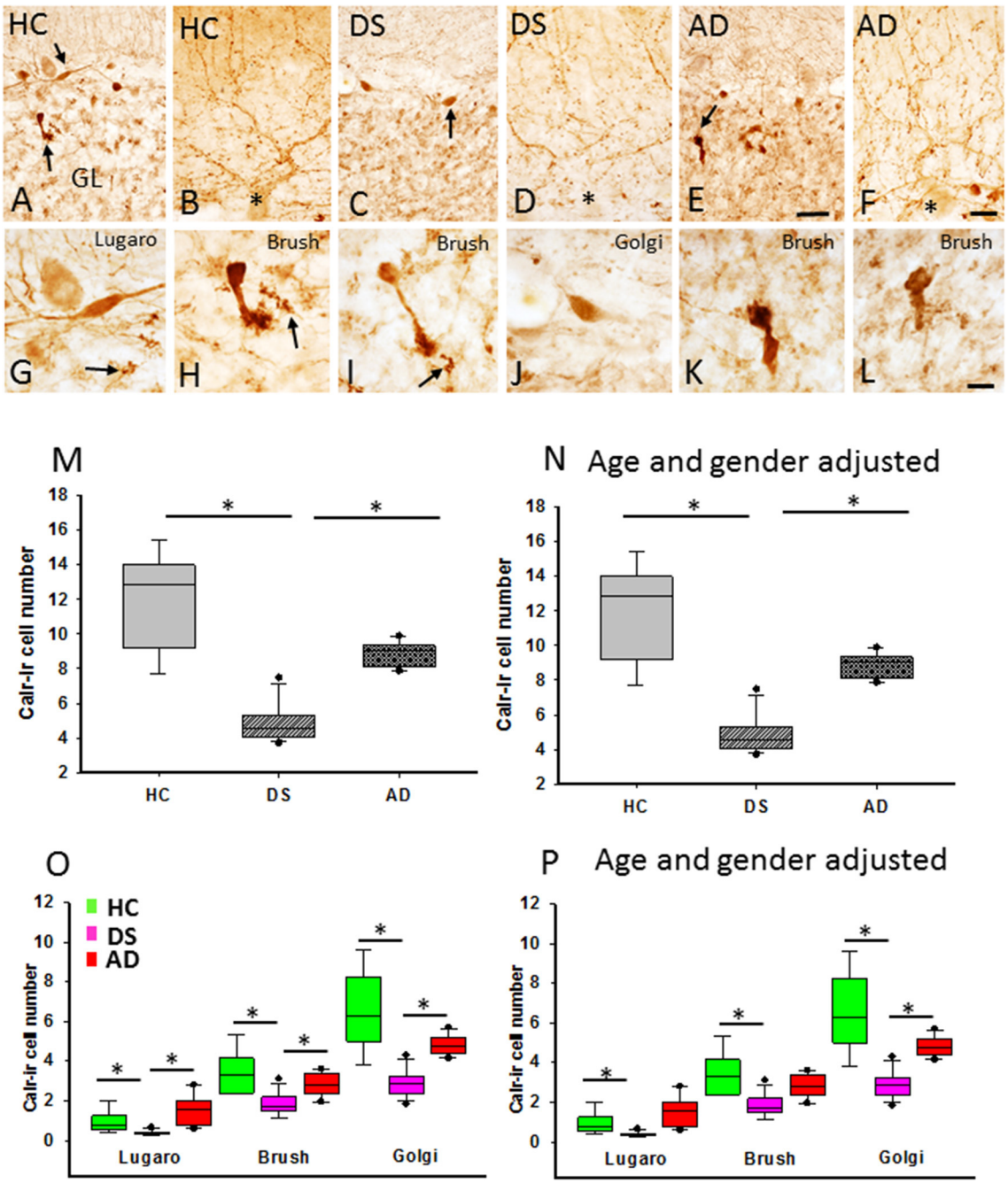

FIGURE 9 | Photomicrographs showing Calr-ir cells **(A,C,E)** and beaded climbing fibers **(B,D,F)** in the GL and ML, respectively, in a 66-year-old female HC **(A,B)**, 46-year-old male with DSD+ **(C,D)**, and a 72-year-old female with AD **(E,F)** case. Note the reduction of Calr-ir cells in DS **(C)** and AD **(E)** compared to HC **(A)**. High-power images of an elongated Lugaro **(G)** shown in **(A)** (upper arrow) and a unipolar brush cell **(H)** (lower arrow in **A**) from a HC, Golgi cell **(J)** shown in **(C)** (arrow) and a unipolar brush interneuron in DS **(I)**, as well unipolar brush cells (**K**: indicated by an arrow in **E**; and **L**) in an AD case. Note the presence of GL Calr-ir MF rosettes in **(G–I)** (arrows). Boxplots showing a significant reduction in Calr-ir cell numbers in DS compared to HC (**M**; Kruskal–Wallis, $p < 0.001$) and AD (**M**; Kruskal–Wallis, $p < 0.002$), even after adjusting for age and gender (**N**; ANCOVA, $p > 0.005$). The number of Calr-ir Golgi, unipolar brush, and Lugaro cells was significantly reduced in DS compared to HC (**O**; Golgi and brush: Kruskal–Wallis, $p < 0.01$; Lugaro: Krukal–Wallis, $p < 0.001$) and AD (**O**; Golgi and brush: Kruskal–Wallis, $p < 0.01$; Lugaro: Kruskal–Wallis, $p < 0.001$). Age and gender adjustment revealed a significant reduction in the Golgi, brush, and Lugaro Calr-ir cell number in DS compared to HC, and only Golgi Calr-ir cell numbers were also reduced compared to AD **(P)**. CF, climbing fibers; MF, mossy fibers. Asterisks in (**B,D,F**) indicate PCs. Scale bars: **(E)** = 50 μm applies to **(A,C)**, **(F)** = 25 μm applies to **(B,D)**, and **(L)** = 10 μm applies to **(G–K)**. * denotes significance between groups.

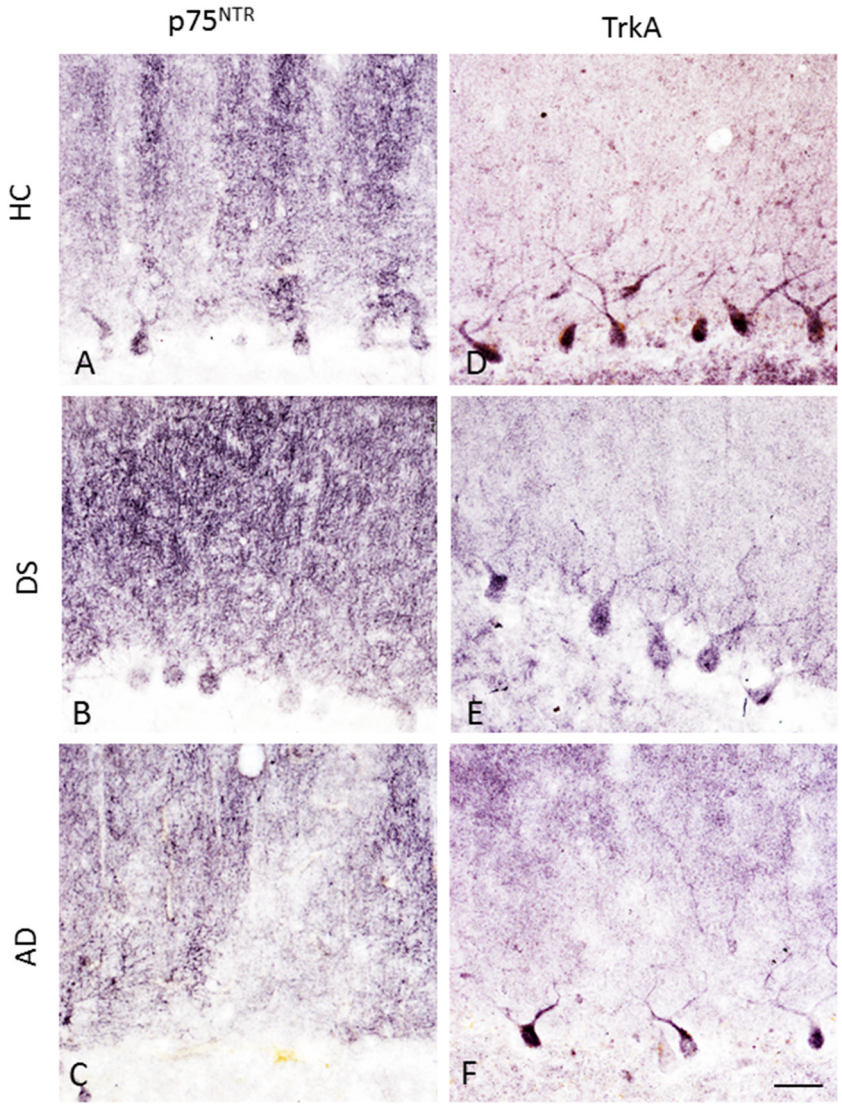

FIGURE 10 | Photomicrographs showing $p75^{NTR}$-ir PC soma, dendritic arbors and axons in a 66-year-old female HC **(A)**, 47-year-old female with DSD– **(B)**, and a 79-year-old female with AD **(C)** case. In contrast, TrkA immunostaining was mainly seen in PC soma with a few positive dendritic branches in a 84-year-old female HC **(D)**, 47-year-old female with DSD– **(E)**, and a 83-year-old female with AD **(F)** case. Scale bar = 50 μm in **(A–F)**.

was increased in DS compared to AD. When adjusted for age and gender DS still displayed a greater $A\beta_{42}$ plaque load than HC and AD with no difference in APP/Aβ plaque load among groups, suggesting that $A\beta_{42}$ plaques in the cerebellum develop at different rates in DS than in AD. Likewise, the ratio of $A\beta_{42}$:APP/Aβ plaque load was greater in DS compared to AD suggesting an accelerated production of $A\beta_{42}$ or higher levels of Aβ N-terminal truncation that precludes recognition by the APP/Aβ 6E10 antibody (Kummer and Heneka, 2014; Thal et al., 2015) a major Aβ component in the cerebellum in DS (Lalowski et al., 1996). However, adjusting for age and gender revealed no significant difference in $A\beta_{42}$:APP/Aβ plaque ratio between groups. The observation that the DS cerebellum contains early stage diffuse non-neuritic plaques (Thal et al., 2006; Catafu et al., 2016) with a filamentous appearance compared to more advanced neuritic plaques reported in the neocortex of both DS and AD (Hof et al., 1995; Nelson et al., 2012; Perez et al., 2019), suggests regional differences in plaque development between these disorders. Unlike sporadic AD (Braak and Braak, 1997; Thal et al., 2002; Verdile et al., 2004), DS amyloid cerebellar plaques remain diffuse even at older ages, suggesting that trisomy APP overexpression differentially affects plaque formation and maturation within different brain regions in DS. Perhaps the lack of neuritic core plaques plays a role in the absence of tau pathology in the cerebellum in DS (present findings; Mann and Jones, 1990; Cole et al., 1993; Li et al., 1994; Davidson et al., 2018) and AD (Aikawa et al., 1985; Azzarelli et al., 1985; Joachim et al., 1989; Mann and Jones, 1990; Li et al., 1994; Mann et al., 1996;

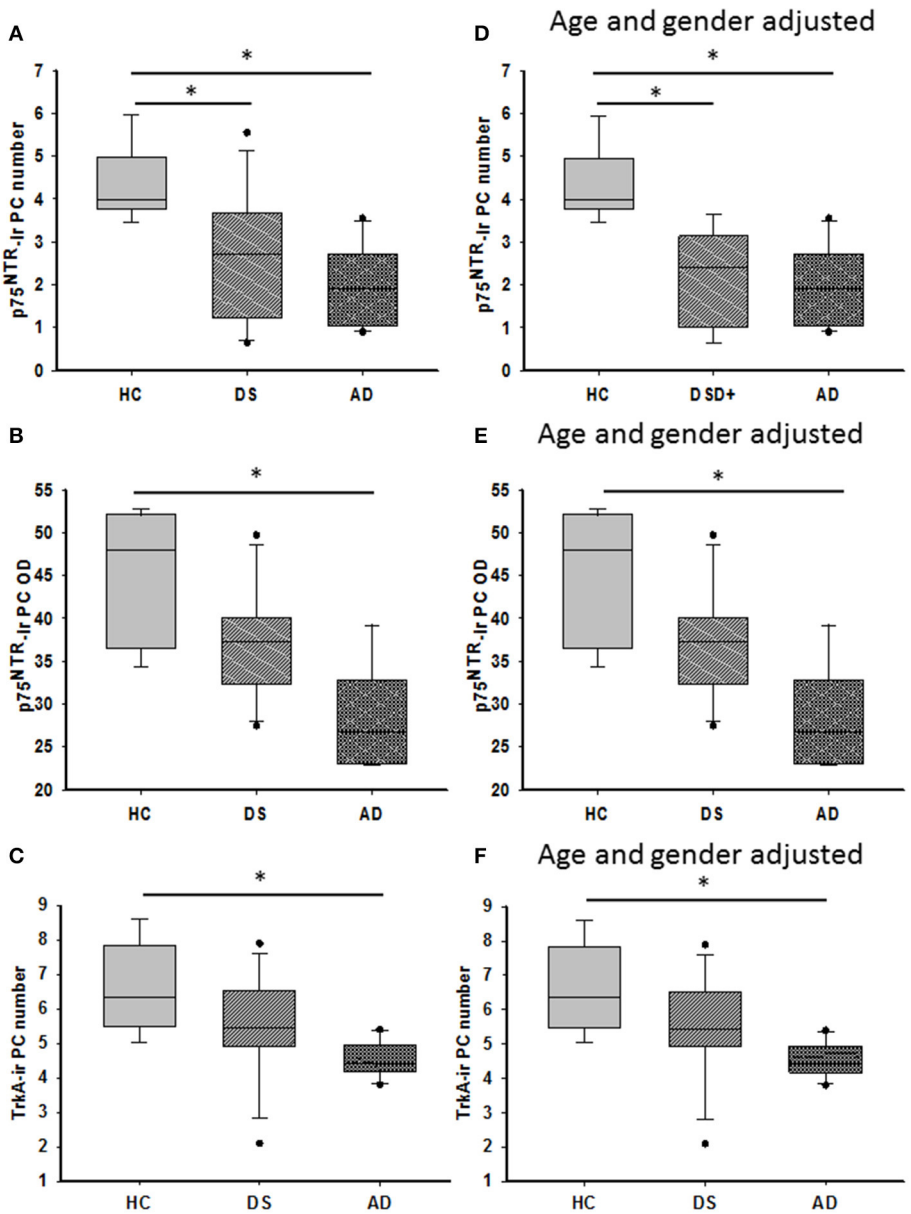

FIGURE 11 | Boxplots showing a significant reduction in the number of $p75^{NTR}$ positive PCs in both DS (**A**; Kruskal–Wallis, $p = 0.03$) and AD (**A**; Kruskal–Wallis, $p = 0.001$) compared to HC. Adjusting for age and gender revealed statistically similar results when DSD– cases were removed from the analysis (**D**; ANCOVA, $p < 0.011$). Optical density (OD) measurements of $p75^{NTR}$-ir PC soma revealed significantly higher values in HC compared to AD (**B**; Kruskal–Wallis, $p = 0.009$) but not different from subjects with DS. Similar statistical findings were found when adjusted for age and gender (**E**; ANCOVA, $p < 0.011$). Boxplots revealed a significant reduction in the number of TrkA-ir PCs in AD compared to HC but not in DS (**C**; Kruskal–Wallis, $p < 0.05$) even after adjusting for age and gender (**F**; ANCOVA, $p < 0.001$). * denotes significance between groups.

Zhu et al., 2019). Interestingly, the morphology and location of amyloid plaques seen in the DS cerebellum is similar to that described in early-onset familial AD caused by a genetic mutation of the Presenilin-1E280A gene (Sepulveda-Falla et al., 2012).

Quantitation of cerebellar PCs stained for H&E or cresyl violet was similar across groups even when adjusted for age and gender supporting prior findings showing no difference in PC soma numbers between AD and HC cases (Andersen et al., 2012; Stepień et al., 2012; Mavroudis et al., 2013, 2019; Tabatabaei-Jafari et al., 2017). Surprisingly, we found a significantly higher number of H&E compared to cresyl violet stained PCs in DS and AD but not in HC cases. Perhaps, this disparity is related to alterations in the cellular milieu underlying the affinity of each histochemical stain. In this regard, cresyl violet labels mRNAs located in Nissl bodies, whereas H&E stains cytoplasm, nuclei, and organelles. The present results derived from the cresyl violet

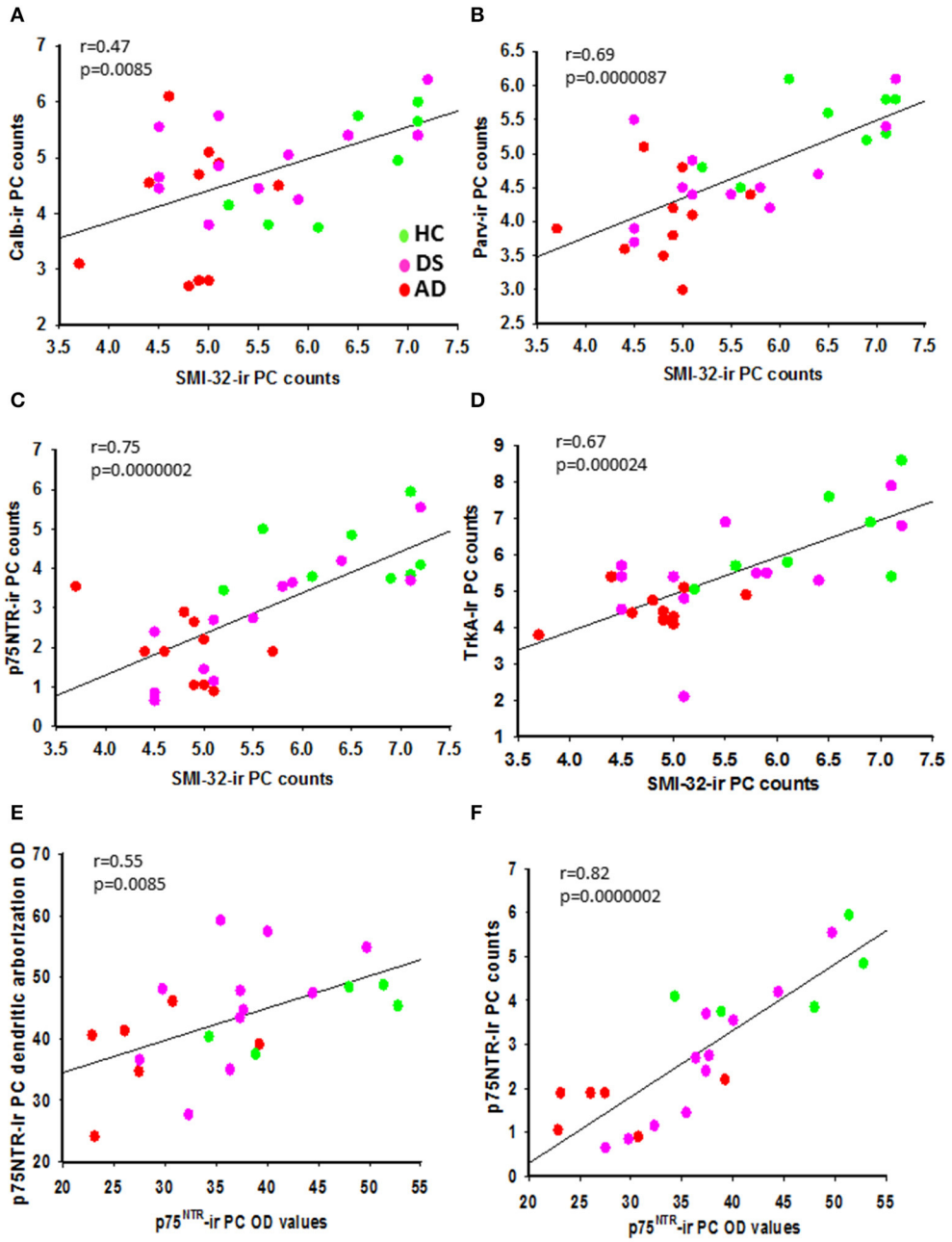

FIGURE 12 | Linear regression analysis revealed significant positive correlations between SMI-32 and Calb- (**A**; $r = 0.47$, $p = 0.085$), Parv- (**B**; $r = 0.69$, p 0.0000087), p75^{NTR}- (**C**; $r = 0.75$, $p = 0.0000002$), and TrkA-ir PC counts (**D**; $r = 0.67$; $p = 0.000024$) across the three groups. Significant positive correlations were seen between p75^{NTR}-ir PC soma and p75^{NTR}-ir PC dendritic arborization OD values (**E**; $r = 0.55$; $p = 0.0085$) and p75^{NTR}-ir PC counts (**F**; $r = 0.82$; $p = 0.0000002$) across groups.

staining suggest mRNA defects in PCs in DS and AD that are not found in HCs. These findings also suggest that there is not a frank loss of PCs in the cerebellum of adults with DS. By contrast, others using cresyl violet and H&E stains report a significant decrease in PC density in AD compared to controls (Fukutani et al., 1996; Wegiel et al., 1999; Sjöbeck and Englund, 2001; Mavroudis et al., 2010), and that PC loss in familial AD (FAD) was greater than in sporadic AD (Fukutani et al., 1997). The discrepancy between the present and earlier findings may be due to a variation in counting procedures and/or cohort. By contrast, we found a highly significant decrease in mean number of SMI-32-ir PCs in DS and AD compared to HC, when adjusted for age and gender. SMI-32 is a well-characterized antibody raised against non-phosphorylated-high-molecular

weight neurofilament proteins (NFH). Neurofilament proteins are cytoskeletal polymers found predominantly in axons that are essential for axonal maintenance and rate of action potential propagation (Sternberger and Sternberger, 1983; Burianová et al., 2015). While our study appears to be the first to describe a decrease in SMI-32 in the cerebellum of an adult with DS, similar decreases are reported in the cortex (Morrison et al., 1987; Hof and Morrison, 1990; Hof et al., 1990; Bussière et al., 2003; Ayala-Grosso et al., 2006; Thangavel et al., 2009) and the hippocampus in AD (Cork et al., 1986; Vickers et al., 1992, 1994; Thangavel et al., 2009) as well as in the brains of normal aged humans (Vickers et al., 1994). Reductions in SMI-32 staining in cortical neurons are associated with an increase in phosphorylation of the neurofilaments that contribute to NFT formation in AD (Cork et al., 1986; Hof et al., 1990; Morrison and Hof, 2002; Veeranna et al., 2011; Vickers et al., 2016). Unlike SMI-32, which reveals non-phosphorylated NFH in PCs, none were SMI-34 positive for phosphorylated high molecular weight neurofilaments in DS, AD, or HC. However, it is known that non-phosphorylated NFH epitopes become increasingly phosphorylated during the aging process (Burianová et al., 2015) and abnormal hyperphosphorylation is considered a trigger for neurofilament accumulation associated with neurodegeneration (Petzold, 2005). Here, we found a greater number of phosphorylated compared to non-phosphorylated NFH torpedoes in DS, AD, and HC subjects. Moreover, the number of GL phosphorylated NFH torpedoes was significantly increased in AD compared to DS, which correlated positively with age and negatively with Aβ plaque load across groups, suggesting aging, but not Aβ pathology, as a factor in the formation of phosphorylated NFH torpedoes. PC axonal torpedoes, which consist of disordered phosphorylated and non-phosphorylated neurofilaments, are thought to underlie defects in axonal transport (Jung et al., 2000; Cleveland and Rothstein, 2001; Robertson et al., 2002; Liem and Leung, 2003; Louis et al., 2012; Didonna and Opal, 2019). The mechanism(s) that trigger PC axonal hyperphosphorylation remain unclear. It is possible that PC torpedoes and hypertrophic axons represent compensatory responses (Kemp et al., 2016) due to neuronal/axonal injury (Petzold, 2005). Together these findings suggest that the reduction of SMI-32 positive PCs and the presence of phosphorylated and non-phosphorylated NFH axonal torpedoes are indicative of alterations in the PC cytoskeleton in DS and AD. Whether PC torpedoes are an age-related phenomenon, a response to injury or driven by genetic or epigenetic factors in DS and AD requires further investigation. Interestingly, alterations in cerebrospinal fluid and blood neurofilament levels are potential diagnostic/prognostic biomarkers for neurodegenerative diseases, including DS (Rafii et al., 2015; Strydom et al., 2018; Fortea et al., 2020) and AD (Jin et al., 2019; Raket et al., 2020).

Purkinje cells are phenotypically characterized by Calb and Parv. These CBPs regulate calcium levels either directly or indirectly enabling (de)sensitization of calcium channels controlling calcium entry into cells to maintain cerebellar function (Bastianelli, 2003). The present study found no difference in the number of Calb immunolabeled PCs and OD values among groups supporting previous report (Stepień et al., 2012). By contrast, a loss of Calb-ir neurons has been reported in the hippocampus (McLachlan et al., 1987; Stefanits et al., 2014), cerebral cortex (Ichimiya et al., 1988) and nucleus basalis of Meynert (Ichimiya et al., 1989; Riascos et al., 2011) in AD. In addition, there is an age-related decrease in PC Calb protein and mRNA levels in humans (Iacopino and Christakos, 1990; Gattoni and Bernocchi, 2019) and rodents (Iacopino and Christakos, 1990; Amenta et al., 1994; Kishimoto et al., 1998). The present findings revealed age as a cofactor underlying changes in Calb containing PCs. Functionally, neuronal expression of Calb confers resistance to neurodegenerative processes during normal aging (Geula et al., 2003) and AD (Riascos et al., 2011). For example, Calb-containing cholinergic forebrain neurons are resistant to phosphorylated tau accumulation and tangle formation in AD (Riascos et al., 2011). In contrast, Parv-ir PC counts were significantly reduced in DS (with and without dementia) and in AD compared to HC cases when adjusted for age and gender (present study). Furthermore, we found fewer ML Parv-ir interneurons (stellate and basket cells) in DS than in AD and HC cases. Since Parv-positive PCs, stellate and basket cells also contain the inhibitory neurotransmitter GABA (Schwab et al., 2013), it is possible that these neurons are more vulnerable to pathological insults in DS. Unlike Calb, no differences in cerebellar Parv-ir neuron number and expression were reported between the young and elderly humans (Satoh et al., 1991), suggesting the decrease in Parv-ir PCs is not age-dependent. However, a reduction in PC Parv immunoreactivity (Stepień et al., 2012) and cell size (Satoh et al., 1991) has been described in AD compared to controls. Decreases in PC Parv mRNA levels and Parv-ir neuron numbers occur in people with autism (Soghomonian et al., 2017), while an increase in cerebellar Parv levels were found in the schizophrenic brain (Vidal-Domènech et al., 2020). These observations indicate that Parv cells are vulnerable in various neurologic disorders, whereas Calb is associated with cellular resistance in the face of neuropathologic diseases (Fairless et al., 2019).

While Calr shares extensive homologies with Calb, the former is observed in a separate population of neurons in the cerebellar cortex. Here, we found Calr positive GABAergic inhibitory Golgi and Lugaro and excitatory unipolar brush cells only in the granular cell layer of the cerebellum. Brush cells are classified in two functionally and chemically distinct subclasses: Type I contain Calr, while Type II are characterized by expression of mGluR1α (Martina and Sekerková, 2016). Based upon these phenotypes, most of the brush cells described here are likely Type I. We found a significant reduction in all three Calr containing cell types in DS compared to HC, while no differences were observed between AD and HC cases. There was a strong correlation between these interneuronal subtypes and Parv-ir interneurons within the ML, possibly due to their close synaptic interrelationships. Brush cells establish contacts upon afferent mossy fibers, which are modulated by the Golgi cells, and send apical dendrites to the ML, which receive inputs from the basket and Lugaro cells (Geurts et al., 2003). Furthermore, a strong association was found between $A\beta_{42}$ plaque load in the ML and low numbers of Parv- and Calr-ir Golgi interneurons

across groups suggesting a possible neurotoxic amylogenic effect. However, human and animal studies suggest that Calr and Calb provide resistance to tau and beta amyloid pathology (Riascos et al., 2011). Since cerebellar PCs contain both Calb and Parv (Baimbridge et al., 1992), this population may be less susceptible to calcium dehomeostasis and neurodegeneration (Arbel-Ornath et al., 2017). We found a reduction in Parv-positive PCs in DS and AD compared to HC and a loss of Parv- and Calr-ir interneurons in DS compared to AD and HC, suggesting that cerebellar CBP circuits are more compromised in DS than AD. Interestingly, Nanostring genomic data derived from the frontal cortex of adults with DS revealed significant decreases in Parv and Calr transcripts compared to HC and AD (unpublished data). At what stage CBP defects occur during the development of the DS brain requires further investigation. Overall, the present data suggest that neuronal calcium dysregulation plays a role in GABAergic inhibitory neurotransmission in DS.

Cerebellar PCs are also characterized by the expression of the neurotrophin protein NGF (Shelton and Reichardt, 1986) and its cognate low affinity $p75^{NTR}$ and high affinity TrkA receptors (Cohen-Cory et al., 1991; Mufson et al., 1991; Hock et al., 1998; Triaca et al., 2016). NGF binds to its TrkA receptor, activating signal transduction pathways key for neuronal survival (Kaplan and Miller, 2004), while $p75^{NTR}$, a modulator of NGF/TrkA binding (Kaplan and Miller, 2004), is associated with cell death (Mufson et al., 2019). Our quantitative analysis revealed a significant reduction in the number of TrkA-ir PCs in AD compared to HC but not DS. AD cases displayed a 31% and DS a 14% reduction in TrkA-positive PCs compared to HC. By contrast, there were 47% fewer TrkA-ir neurons within nucleus basalis of subjects with DS than aged controls (Sendera et al., 2000). We also found a significant reduction in the number of $p75^{NTR}$-ir PCs in AD and DS compared to HC cases. $P75^{NTR}$ PC soma OD measurements were significantly reduced in AD compared to HC but not in DS. However, when the data were adjusted for age and gender, the number of TrkA and $p75^{NTR}$ containing PC cells decreased in AD compared to HC but only $p75^{NTR}$ PCs in DSD+ compared to HC. By contrast, it has been reported that TrkA and $p75^{NTR}$ mRNA levels are not altered in the AD cerebellum compared to controls (Hock et al., 1998). Discrepancy between protein and mRNA is frequently reported and could explain these contradictory findings (Gygi et al., 1999; Washburn et al., 2003). Although we did not find an association between cerebellar APP/Aβ and $Aβ_{42}$ load with numbers of TrkA or $p75^{NTR}$ labeled PCs in DS, a strong negative relationship was observed between APP/Aβ load and $p75^{NTR}$ but not in the number of TrkA containing PCs in AD. TrkA binds APP preventing its cleavage into Aβ peptides (Costantini et al., 2005; Triaca et al., 2016) and activated Trks suppress apoptotic pathways induced by the binding of Aβ to $p75^{NTR}$ (Matrone et al., 2008, 2009, 2011). Perhaps, the genetic overexpression of APP in DS leads to an increase in APP/TrkA binding (Triaca et al., 2016), protecting PCs from apoptosis activated *via* $p75^{NTR}$. Others have reported a lower expression of $p75^{NTR}$ in PC neurons in healthy adults (Shelton and Reichardt, 1986; Koh and Loy, 1989; Cohen-Cory et al., 1991) with re-expression induced post-injury (Sofroniew et al., 2001). These observations together with the present findings showing similar numbers of PCs positive for TrkA and $p75^{NTR}$ in DS, but decreases in AD compared to HC subjects, indicate that cerebellar NGF metabolism is less affected in DS than in AD. Examination of other components of NGF metabolism, which have been studied in DS (Iulita et al.,

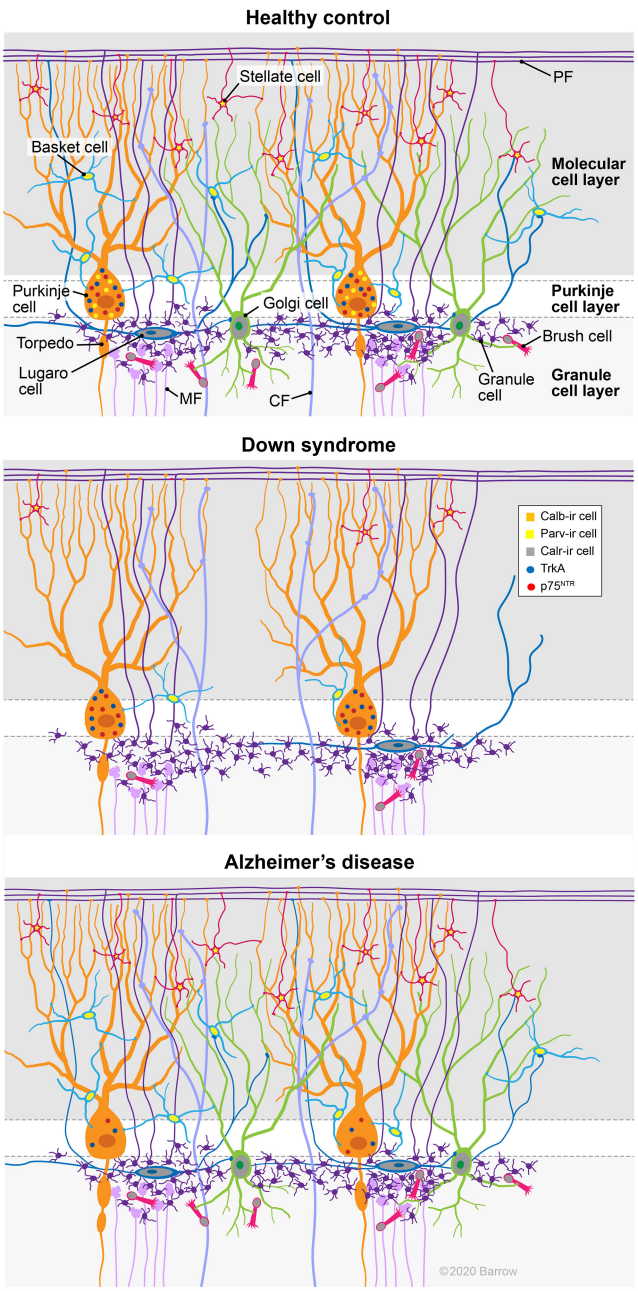

FIGURE 13 | Summary diagram depicts cellular alterations in DS and AD compared to HC cases. Note the reduction of Parv containing PCs as well as Parv (basket/stellate) and Calr (Golgi, Lugaro, and brush) positive interneurons in the cerebellar cortex of an adult with DS. By contrast, TrkA and $p75^{NTR}$-ir PC numbers were lower in AD compared to HC, but not in DS. CF, climbing fibers; MF, mossy fibers; PF, parallel fibers. Used with permission from Barrow Neurological Institute, Phoenix, Arizona.

2014) and AD (Mufson et al., 2019) are required to decipher the molecular mechanisms underlying the role that NGF and its receptors play in PC dysfunction in DS.

The findings reported in the present study are summarized in **Figure 13**. Here, we provide the first evidence of a reduction in Parv containing PCs as well as Parv (basket/stellate) and Calr (Golgi, Lugaro, and brush)-positive interneurons in the cerebellar cortex of adults with DS. In contrast, TrkA and $p75^{NTR}$-ir PC number was lower in AD compared to HC but not in DS. Although, we did not find NFTs and neuritic plaques in the cerebellum of adults with DS, deficits in CBPs and/or NGF metabolism in the cerebellar connectome may play a role in the cognitive and motor deficits reported in DS and AD. Overall, we have shown extensive cellular degenerative events in the cerebellum that should be considered as potential targets for therapeutic intervention in DS and AD.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

SP and EM: study concept and design, analysis and interpretation of data, and study supervision. JM and MM-A: acquisition of data. JM, SP, and EM: drafting of the article. All authors had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnagi.2021.645334/full#supplementary-material

Supplementary Figure 1 | Photomicrographs demonstrating the lack of Purkinje cell (PC) TrkA (**A**, arrows) and $p75^{NTR}$ (**C**, arrows) immunostaining after omission of each antibody compared to antibody reactiviy for TrkA **(B)** and $p75^{NTR}$ **(D)** in a female 47-year-old DSD– case. Lower panels demonstrate that the current TrkA antibody does not immunostain neurons containing TrkB. Similar to previous studies (Mufson and Kordower, 1989; Mufson et al., 1989), cholinergic neurons within the nucleus basalis of Meynert (nbM) are TrkA **(E)** and $p75^{NTR}$ **(F)** immunopositive in tissue from a 93-year-old female HC and a 94-year-old female with AD, respectively. **(G,H)** Images showing TrkA immunopositive (dark blue) neurons in the oculomotor/crainal nerve III (**G**) in contrast to the absence of TrkA containing neurons in substantia nigra (SN) pars compacta (Sobreviela et al., 1994, **H**, arrows), which express TrkB, but not TrkA in tissue obtained from a 51-year-old male HC. Findings support the specificity of the TrkA immunostaining of PCs shown in the present study. Sections in **(A,C)** were counterstained with hematoxylin. In **(H)**, the brown pigment is the neuromelanin found in SN neurons. Scale bar: **(H)** = 50 μm applies to **(A–G)**, respectively.

Supplementary Figure 2 | Box plots showing no difference in the ratio between cresyl violet and H&E stained PC numbers between groups (**A**; Kruskal–Wallis test, $p > 0.05$), even after adjusting for age and gender **(B)**. Conversely, the ratio of SMI-32-ir to H&E PC counts in AD, was significantly lower compared to HC (**C**; Kruskal–Wallis, $p = 0.03$). Adjusting for age and gender yielded similar findings **(D)**. *denotes significant differences between groups.

Supplementary Figure 3 | Immunofluorescence images of single labeled Calb (green), Parv (red), and merged (yellow/green) within the cerebellum of a 69-year-old female HC **(A–C)** and a 60-year-old female dementia DS **(D–F)** case. Merged images show CBP dual-labeled PCs (yellow–green) in HC **(C)** and DS **(F)**. Numerous immunofluorescent Parv-ir interneurons were seen in HC **(B)** compared to lesser numbers in DS **(E)**. Immunofluorescence single-labeled Calb (green) **(G)** and SMI-32 (red) **(H)** and merged images of PCs (yellow) **(I)** in a 60-year-old female dementia subject with DS. Note the presence of a rare dendritic torpedo positive for Calb and SMI-32 (white arrows). Scale bars: **(F)** = 50 μm and applies to **(A–E)**; **(I)** = 75 μm and applies to **(G,H)**.

Supplementary Figure 4 | Linear regression analysis revealed a significant positive correlation between $p75^{NTR}$-ir PC soma OD values and SMI-32-ir PC counts (**A**; $r = 0.68$; $p = 0.00038$). TrkA-ir PC soma OD values and number correlated positively (**B**; $r = 0.57$; $p = 0.0053$). Significant positive correlations were found between Parv-ir and Calr-ir interneuron counts (**C**; $r = 0.76$; $p = 0.0000002$) and Calr-ir Golgi cell counts (**D**; $r = 0.74$; $p = 0.0000002$) across the three groups. Calr-ir Golgi interneuron number exhibited a strong positive correlation with unipolar brush (**E**; $r = 0.73$; $p = 0.0000002$) and Calr-ir Lugaro cell counts (**F**; $r = 0.68$; $p = 0.00003$).

Supplementary Figure 5 | Linear regression analysis revealed a significant negative correlation between Parv- and Calr-ir cell counts and amyloid plaque load across groups. Parv-ir interneuron counts were negatively correlated with $A\beta_{42}$ plaque (**A**; $r = -0.74$; $p = 0.0000002$) and APP/Aβ plaque load (**B**; $r = -0.61$; $p = 0.00033$). Calr-ir cell counts negatively correlated with $A\beta_{42}$ plaque load (**C**; $r = -0.70$; $p = 0.0000002$) and to a lesser extent with APP/Aβ plaque (**D**; $r = -0.47$; $p = 0.0082$) load across groups. Calr-ir Golgi cell counts negatively correlated with $A\beta_{42}$ plaque load (**E**; $r = -0.71$; $p = 0.0000002$) but displayed a weaker association with APP/Aβ plaque load (**F**; $r = -0.45$; $p = 0.014$) across groups.

Supplementary Figure 6 | Linear regression analysis revealed a strong significant positive correlation between GL SMI-34-ir torpedo counts and subject age (**A**; $r = 0.62$; $p = 0.0003$) across all groups. Significant negative correlations were seen between NFT Braak scores and counts for SMI-32- (**B**; $r = -0.66$; $p = 0.00007$), $p75^{NTR}$- (**C**; $r = -0.73$; $p = 0.0000002$) and Parv-ir PCs (**D**; $r = -0.59$; $p = 0.00064$). PC TrkA-ir counts (**E**; $r = -0.57$; $p = 0.001$) and $p75^{NTR}$-ir OD values (**F**; $r = -0.57$; $p = 0.006$) correlated negatively with NFT Braak scores across groups.

Supplementary Figure 7 | Linear regression analysis revealed a significant negative correlation between NFT Braak scores and TrkA-ir PC soma OD values (**A**; $r = -0.56$; $p = 0.008$) and Calr-ir cell counts (**B**; $r = -0.58$; $p = 0.00094$) across groups.

Supplementary Table 1 | Case demographics.

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ORIGINAL RESEARCH
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Similar Genetic Architecture of Alzheimer's Disease and Differential *APOE* Effect Between Sexes

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Sex differences have been observed in the clinical manifestations of Alzheimer's disease (AD) and elucidating their genetic basis is an active research topic. Based on autosomal genotype data of 7,216 men and 10,680 women, including 8,136 AD cases and 9,760 controls, we explored sex-related genetic heterogeneity in AD by investigating SNP heritability, genetic correlation, as well as SNP- and gene-based genome-wide analyses. We found similar SNP heritability (men: 19.5%; women: 21.5%) and high genetic correlation (R_g = 0.96) between the sexes. The heritability of *APOE* ε4-related risks for AD, after accounting for effects of all SNPs excluding chromosome 19, was nominally, but not significantly, higher in women (10.6%) than men (9.7%). In age-stratified analyses, ε3/ε4 was associated with a higher risk of AD among women than men aged 65–75 years, but not in the full sample. Apart from *APOE*, no new significant locus was identified in sex-stratified gene-based analyses. Our result of the high genetic correlation indicates overall similar genetic architecture of AD in both sexes at the genome-wide averaged level. Our study suggests that clinically observed sex differences may arise from sex-specific variants with small effects or more complicated mechanisms involving epigenetic alterations, sex chromosomes, or gene-environment interactions.

Keywords: Alzheimer's disease, sex difference, heritability, genetic heterogeneity, genome-wide association study

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INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by progressive memory loss and is the most common form of dementia (Winblad et al., 2016). The majority of cases are the sporadic form of late-onset AD. The estimated prevalence of AD among adults over the age of 65 years in the United States is about 10%, and approximately two-thirds of AD patients are women (Alzheimer's Association, 2020).

Phenotypic differences between sexes in AD have long been noted in various aspects (Dubal, 2020). For example, the cognitive and psychiatric symptoms present differently between women and men (Mielke et al., 2014; Snyder et al., 2016; Pike, 2017; Ferretti et al., 2018; Laws et al., 2018). Longitudinal data suggest greater cognitive decline and hippocampal atrophy rates in women after diagnosis of mild cognitive impairment (MCI) or AD (Hua et al., 2010; Holland et al., 2013; Lin et al., 2015; Gamberger et al., 2017), even though few differences have been reported to date in biomarkers of AD, such as accumulation of amyloid-β and tau proteins (Ferretti et al., 2018). Epidemiologically, the risk and protective factors of AD distribute unequally between the sexes. For instance, there is a higher prevalence of risk factors in postmenopausal women, such as cardiovascular diseases, depression, and sleep disorders; whereas educational attainment and physical activity tend to be higher in men, which are protective factors of AD (Xu et al., 2016; Stephen et al., 2017). In addition, evidence suggests that there are risk factors specific to women, including factors related to reproductive history, pregnancy complications, or hormone replacement therapy (Ferretti et al., 2018; Nebel et al., 2018; Gilsanz et al., 2019). In general, the lifetime risk of developing AD in those aged 65 years or older is twice as high in women than in men (21.2% vs. 11.6%, respectively; Alzheimer's Association, 2020). However, the reasons for these sex differences are not completely clear to date.

As a complex polygenic disease, the etiology of AD may reflect a combination of genetic and environmental effects. In this study, we specifically focused on the genetic architecture that characterizes the genetic factors underlying this heterogeneity in the context of a polygenic framework, considering the collective effects of multiple genetic risk variants (Timpson et al., 2018). Emerging evidence suggests that the effect of the apolipoprotein E (*APOE*) ε4 allele, a major genetic risk factor for AD, is modified by sex (Farrer et al., 1997; Altmann et al., 2014). Notably, a large-scale meta-analysis demonstrated that although the *APOE* ε4 allele confers generally a similar risk of developing AD in women and men aged 55–85 years, noteworthy differences can be found when stratifying patients by age groups (Neu et al., 2017). Specifically, this study found that ε3/ε4 was associated with an increased risk of AD in women compared to men between the ages of 65–75 years. It has also been supported by neuroimaging studies which demonstrated significant *APOE*-by-sex interaction in the distribution of cerebral hypometabolism and changes in cortical thickness (Sampedro et al., 2015), as well as a higher prevalence of *APOE*-ε4-associated cerebral small vessel disease in male AD patients (Finch and Shams, 2016).

Previous studies revealed multiple variants with significant sex-by-genotype interactions in AD (Dumitrescu et al., 2019; Gamache et al., 2020), and genome-wide association studies (GWAS) have identified many AD susceptibility loci in addition to *APOE* (Harold et al., 2009; Lambert et al., 2009; Seshadri et al., 2010; Hollingworth et al., 2011; Naj et al., 2011; Jansen et al., 2019; Kunkle et al., 2019). However, due to low statistical power to robustly detect sex-specific loci in AD after sample stratification, few studies have investigated sex difference effects through a classical GWAS approach to identify variants associated with the AD diagnosis in case-control cohorts (Nazarian et al., 2019). Other study paradigms such as incorporating family-based association design or leveraging neuropathological features as AD endophenotypes have found sex-specific associations (Deming et al., 2018; Dumitrescu et al., 2019; Prokopenko et al., 2020). In addition to detecting sex-specific loci, some studies identified significant sex-specific predictors for AD phenotypes such as neuropathology (Deming et al., 2018), biomarkers (Dumitrescu et al., 2019), and age at onset using polygenic hazard scores (Fan et al., 2020). In the present study, we estimated sex-stratified single nucleotide polymorphism (SNP) heritability of AD and genetic correlation between sexes, in which a large number of common SNPs with small effects contribute additively to phenotypic variation. Although this polygenic model cannot detect sex-specific loci, it allows us to investigate the genetic architecture of AD between sexes with adequate statistical power using the Alzheimer's Disease Genetics Consortium (ADGC) sample. We then examined *APOE* heritability and the odds ratio (OR) for AD with mixed linear models, and lastly performed exploratory sex-stratified genome-wide analyses.

MATERIALS AND METHODS

ADGC Sample

The 2-phase ADGC data include 15 cohorts in both phases with 18,844 and 5,342 individuals, respectively, of European ancestry aged 60 years and above (except 1 AD patient with the age at onset at 58 years old), who were enrolled between 1989 and 2011. The ADGC data also include common covariates (age at onset of AD or age at the first visit for controls, sex, and top 10 principal components) to correct for population stratification. The details of each cohort in phase 1 and phase 2 are shown in **Supplementary Table 1**. Quality control was conducted on genotyping call rate, X-chromosome analysis for sex, and identity by descent for relatedness and sample duplication (Jun et al., 2010; Naj et al., 2011). Genotyped SNPs with low minor allele frequencies (<0.02 for Affymetrix chips or <0.01 for Illumina chips) or violation of Hardy-Weinberg equilibrium (P value $< 10^{-6}$) were excluded. Genome-wide SNP imputation was performed in each cohort using the 1,000 Genomes reference panel and imputed SNPs were removed if imputation *quality* (R^2) < 0.5 (Jun et al., 2010).

Whole-Genome SNP Heritability and Genetic Correlation Estimation

The sex-stratified SNP heritability estimates of AD were calculated as the proportion of phenotypic variance explained by SNPs from the whole genome, implemented by Genome-wide Complex Trait Analysis (GCTA; Yang et al., 2011a). GCTA fits effects of all SNPs simultaneously as random effects and effects of other covariates (age, cohort indicators, and the top 10 principal components) as fixed effects in a mixed linear model. In the regression model, the variance explained by SNPs can be estimated by the restricted maximum

likelihood (REML) approach using the genetic relationship matrix (GRM), which reflects the genetic correlations between individuals (Yang et al., 2010). In our analysis, SNPs with minor allele frequencies >0.01 were retained to estimate the GRM, and related individuals were excluded if individual-pairwise GRM >0.025. The SNP heritability estimates were also partitioned through two independent GRMs into chromosome 19, which harbors the *APOE* region, and the remaining 21 chromosomes (Yang et al., 2011b). The genetic correlation between sexes was estimated using the bivariate REML method (Lee et al., 2012), which implies genetic heterogeneity if it significantly differs from 1.

A total of 7,216 males and 10,680 females were included for both analyses combining cohorts of both ADGC phase 1 and 2, and the statistical power of the genetic correlation analysis was evaluated using the GCTA-GREML power calculator[1]. With the above sample sizes, estimated disease prevalence in the population, the lowest estimated SNP heritability of 0.19 as previously reported (Zhang et al., 2020), type I error rate (α) of 0.05 and the default variance explained by SNP-derived genetic relationships of 2×10^{-5}, the calculated power was 1.0 for both sex-stratified analyses.

We used the AD prevalence estimates to correct ascertainment bias due to oversampled cases in case-control study studies (Lee et al., 2011). As AD accounts for the majority of dementia cases, we estimated AD prevalence by using age-and gender-specific estimates of dementia prevalence in the United States from a systemic meta-analysis, which included 5-year prevalence for those over 60 years of age in males and females (Prince et al., 2013). We re-calculated average prevalence for males and females (**Supplementary Table 2**) weighted by age-and sex-specific annual estimates of the resident population of the United States in 2015 from the United States Census Bureau[2] (Lee et al., 2011). This resulted in a prevalence of 0.055 in males and 0.072 in females. The resulting prevalence information was only used in the GCTA analyses above.

APOE ε4 SNP Heritability Estimation

To estimate heritability attributable to the *APOE* ε4 alleles, we included only the GRM generated from all chromosomes excluding chromosome 19, including the same covariates in the mixed linear model as above. We then calculated the best linear unbiased prediction (BLUP), which is the total genetic effect and residual effect for each individual (Yang et al., 2011a). We regressed residuals generated from BLUP estimation in a linear model on the number of *APOE* ε4 alleles and obtained R^2 for males (N = 6,896) and females (N = 10,150) separately, which is the proportion of the variance of the residuals explained by *APOE* ε4 alleles and denotes the heritability of *APOE* ε4 alleles.

The effect sizes of one and two *APOE* ε4 alleles were also estimated by calculating the ORs between AD and control groups in the logistic regression model. In addition, we studied the ORs in younger and older age groups with a cut-off of 80 years old, which was selected based on our prior analysis that indicated a greater genetic heterogeneity between these age groups, and previous studies that suggested a reduced risk of AD associated with ε4 among the population above 80 years old (Bonham et al., 2016; Neu et al., 2017; Lo et al., 2019a), although data are needed to replicate the results. We also specifically compared the ORs of ε3/ε4 and ε3/ε3 in participants ages 65–75 years, based on the previous publication that reported a higher risk conferred by ε3/ε4 in women than in men in this age group (Neu et al., 2017).

The linear and logistic modeling were computed in R, and the confidence intervals of R^2 were calculated using the CI.Rsq function in the psychometric package for R. The sample sizes were slightly smaller due to missing *APOE* ε4 status for some individuals.

Exploratory SNP-Based GWAS of AD

GWAS of 38,043,082 SNPs were separately performed in males and females using logistic regressions implemented in PLINK 1.9 (Chang et al., 2015). Age at disease onset of AD (or age at the first visit for the control group), cohort indicators, and the top 10 principal components were included as covariates. Subjects with individual-pairwise GRM > 0.1 were excluded from analyses to ensure sample independence (Wray et al., 2013). A total of 8,682 males (4,010 cases and 4,672 controls) and 12,772 females (5,705 cases and 7,067 controls) were included combining cohorts of both ADGC phase 1 and 2. Significant SNPs with genome-wide p-value $< 5 \times 10^{-8}$ were obtained, and clumped using the European reference panel of the 1,000 Genomes Project phase 3 (released in May 2013; Auton et al., 2015), to remove correlated SNPs with LD $r^2 > 0.1$ within 250 kb of the top SNP using PLINK 1.9 to obtain LD-independent SNPs (Chang et al., 2015). We used the METAL software to implement Cochrane's Q test for heterogeneity for each SNP between male and female GWAS (Willer et al., 2010).

Gene-Based Analyses

To reduce the number of tests conducted in SNP-based GWAS and aggregate the small effect of each SNP within a gene, we performed sex-stratified gene-based analyses using MAGMA v1.08 implemented in FUMA v1.3.6a (Watanabe et al., 2017). The gene-based p-value was calculated based on the mean of the summary statistic (χ^2 statistic) of GWAS for the SNPs in a gene (de Leeuw et al., 2015; Watanabe et al., 2017). SNPs with minor allele frequencies ≥0.01 in the European reference panel of 1,000 Genomes Project were included. The distance between two LD blocks < 250 kb was merged into a locus. In our analyses, SNPs within the genes were mapped to 18,338 loci (genes). The significant p-value was determined by the Bonferroni method, which divides 0.05 by the number of genes (19,151) resulting in 2.61×10^{-6}. The sex-stratified gene-based analyses using summary statistics from sex-stratified GWAS were performed to obtain significant genes for males and females.

Verification of Results With Matched Female Sub-cohort

As the sizable difference in sample sizes between the two sex strata led to discrepancy of statistical power (male-to-female

[1]https://shiny.cnsgenomics.com/gctaPower

[2]https://www2.census.gov/programs-surveys/popest/datasets/2010-2015/national/asrh/nc-est2015-agesex-res.csv

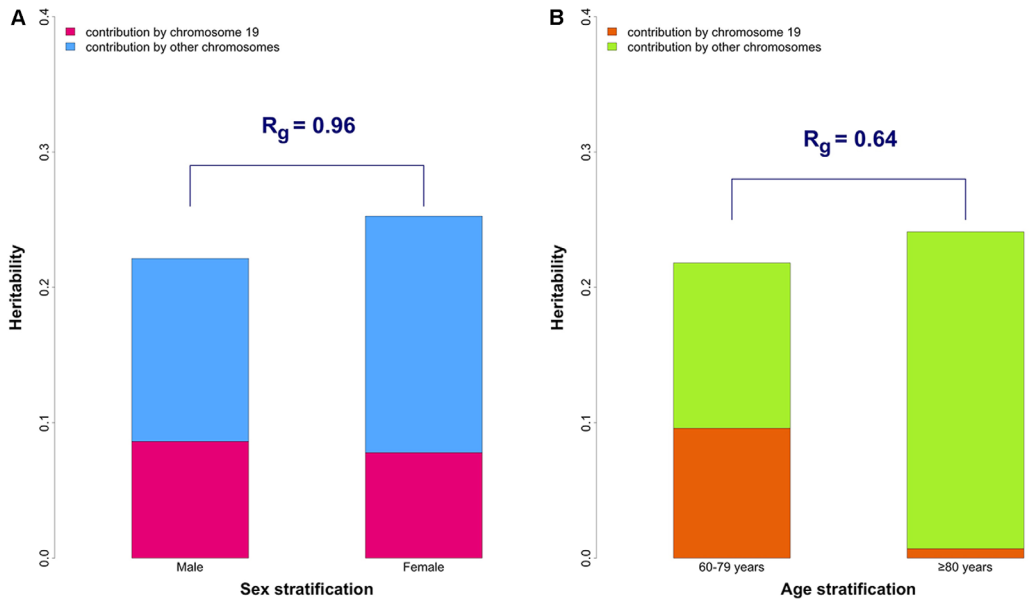

FIGURE 1 | The overall and partitioned heritability estimates in combined phase 1 and 2 samples. **(A)** Comparison between sexes. The heritability estimates were 19.5% (95% CI: 9.9–29.1%) and 21.5% (95% CI: 15.0–28.1%) in male (N = 7,216) and female (N = 10,680) groups, respectively. Heritability estimates of chromosome 19 are 8.6% (95% CI: 5.9–11.4%) in males and 7.8% (95% CI: 5.8–9.8%) in females. The genetic correlation (R_g) between the two sexes was 0.96 (p-value for H_0: R_g = 1 was 0.42). **(B)** Comparison between younger (60–79 years old) and older (≥ 80 years old) groups (from our previously published data; Lo et al., 2019b). The genetic correlation (R_g) between the two age groups is 0.64 which significantly differs from 1 (p-value for H_0: R_g = 1 was 0.043).

ratio: 1:1.5) and might bias our analysis on sex difference, we formed a female sub-cohort with matched numbers of cases and controls as the male cohort by random selection, and repeated the age-and sex-stratified analyses on the *APOE*-ε4 effects, as well as the genome-wide SNP and gene-based analyses.

RESULTS

Whole-Genome SNP Heritability and Genetic Correlation Estimates

The whole-genomic heritability estimates of AD were 19.5% (95% CI: 9.9–29.1%) in males and 21.5% (95% CI: 15.0–28.1%) in females respectively, and overall 20.6% (95% CI: 16.4–24.8%) among the combined ADGC phase 1 and 2 cohorts.

The heritability estimates partitioned by chromosome 19 and other chromosomes are shown in **Figure 1A** and **Supplementary Table 3**. The contribution of chromosome 19 was similar in males and female, which is in contrast to the results that we previously reported in age-stratified analysis (**Figure 1B**).

The genetic correlation (R_g) between males and females was 0.96 (p-value for H_0: R_g = 1 was 0.42) for the whole genome. The results were unchanged after excluding chromosome 19 (R_g = 0.96, p-value for H_0: R_g = 1 was 0.43), suggesting overall genetic homogeneity between sexes in AD.

APOE ε4 SNP Heritability Estimation

The heritability of AD due to *APOE* ε4 was estimated to be 9.7% (95% CI: 8.4–11.0%) in males (N = 6,896), 10.6% (95% CI: 9.5–11.8%) in females (N = 10,150), and 10.2% (95% CI: 9.4–11.1%) in the whole sample.

APOE ε4-associated risk of AD was similar between males and females, with ORs of 3.85 (95% CI: 3.40–4.37) for AD in males and 4.10 (95% CI: 3.71–4.52) in females with one ε4 allele, and ORs of 13.24 (95% CI: 9.69–18.26) and 11.59 (95% CI: 9.19–14.76) in males and females with two ε4 alleles compared to non-carriers. Stratification by age-at-onset of AD demonstrated higher *APOE* ε4-associated ORs in the younger group (onset at 60–80 years old) compared to the older group (onset later than 80 years old) in both males and females as shown in **Supplementary Table 4**, suggesting a higher genetic risk conferred by *APOE* ε4 alleles among younger patients. Consistent results were seen in the female sub-cohort with matched case and control numbers as the male cohort (**Supplementary Table 5**).

Although we did not observe significant differences between men and women in *APOE* ε4-associated ORs in this age group, the subgroup analysis comparing ε3/ε4 and ε3/ε3 among the narrower age group of 65–75 years demonstrated a higher risk in females (OR: 5.93, 95% CI: 4.88–7.22) than males (OR: 3.51, 95% CI: 2.78–4.44), which was also observed in the female sub-cohort (OR: 5.92, 95% CI: 4.68–7.51). No notable sex differences were found in the other age or *APOE* genotype subgroups.

SNP-Based GWAS of AD

Significant SNPs in the *APOE* region and *BIN1* were identified, which have been reported in previous GWAS. No novel SNP was detected in either sex from SNP-based GWAS,

and no genome-wide significant ($p < 5 \times 10^{-8}$ in GWAS) LD-independent SNPs with significantly different effect sizes between sexes (heterogeneity $p < 0.05$) were identified by heterogeneity Cochrane's *Q* tests (**Supplementary Table 6**).

Gene-Based Analyses

Sex-stratified gene-based analyses were then performed in 8,682 males and 12,772 females. Apart from *APOE, APOC1, TOMM40, PVRL2, BCL3, and BCAM* on chromosome 19, no novel genome-wide significant gene was identified (**Figure 2**, **Table 1**). *BCAM* was significant among females only, but the Cochrane's *Q* test demonstrated no sex-related heterogeneity for the SNPs within this gene (range of heterogeneity *p*-values: 0.27–0.91). In addition, *BCAM* was not significant in the female sub-cohort (**Supplementary Figure 1**, **Supplementary Table 7**), consistent with the result from the heterogeneity *Q* test using the full sample showing no sex-related effect.

DISCUSSION

The present study demonstrated a largely similar genetic basis of AD between males and females. Results of partitioned SNP heritability showed similar genetic effects of both the *APOE*-harboring chromosome 19 and the remaining 21 chromosomes in AD in males and females, as well as a high genetic correlation, which captures the genome-wide architecture of AD. These results indicate that the overall genetic underpinnings and architecture of AD are similar across sexes, in contrast to the genetic heterogeneity across age as identified previously (Lo et al., 2019a).

Targeted analyses on *APOE* ε4 alleles demonstrated no disparity in heritability of AD between sexes, but higher heritability in the early-onset groups of both sexes (Lo et al., 2019a). We did not find sex differences in *APOE* ε4 allele-associated genetic risk of AD using the entire age group or subgroups of 60–80 and >80 years old, but specific subgroup analysis replicated the previously reported finding that ε3/ε4 confers higher risk in women than men aged 65–75 years (Neu et al., 2017). We noted that this was not a completely independent replication because up to 58.5% of our samples (n = 21,454) overlap with 21.6% of the sample (n = 57,979) in the prior report (Neu et al., 2017). This may reflect intricate interactions between age, sex, and *APOE*-ε4, involving pleiotropy, tauopathy, and estrogen response of *APOE* (Riedel et al., 2016).

As a pilot study, the exploratory sex-stratified GWAS did not identify any new loci with significant sex-related heterogeneity. The *APOE-APOC1-TOMM40* region in chromosome 19 was significantly associated with AD in both sexes, although substantial sex-related changes in lipid metabolism may be associated with this region. Emerging data support the role of APOE lipidation and brain lipid transport in the development of AD (Husain et al., 2021). It is evident that estrogen regulates the expression and synthesis of APOE, and APOE facilitates the neuroprotective effects of estrogens and androgens, suggesting the sex hormone-APOE interaction may underlie the sex difference in AD (Gamache et al., 2020).

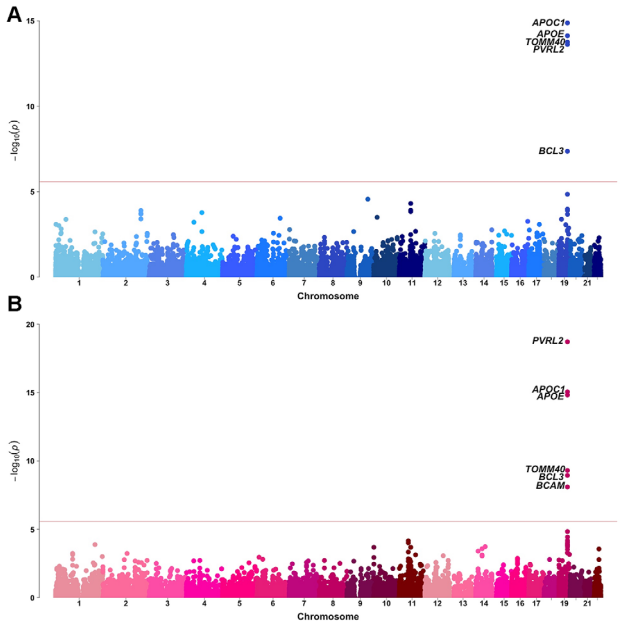

FIGURE 2 | Manhattan plots of MAGMA-annotated genes in Alzheimer's disease genetics consortium (ADGC) combined phase 1 and phase 2 samples of **(A)** male and **(B)** female strata. The red line denotes the gene-based genome-wide significance level of *P*-value = 2.61×10^{-6}.

IMPLICATIONS

As a multifactorial disease, sex-related phenotypic diversity in AD has been noted in multiple studies. The observed differences have been described to arise from combined effects of genetic, epigenetic, cellular and, environmental mechanisms leading to a heterogeneous disease etiology, especially for late-onset AD. In the present study, we found a similar genetic architecture of AD between women and men, which implies that effect sizes of sex-difference variants are likely to be small and detecting these variants through a classical GWAS approach requires a larger sample than the current one. It is likely that age-by-sex interactions in AD further complicate detecting sex-difference variants. Additionally, there is a likely crucial role for gene-environmental interaction at multiple epigenetic levels for the observed sex differences in AD (Guo et al., 2021). Further systematic studies on epigenomic, gene expression, and immunomic profiling, as well as the inclusion of a larger spectrum of environmental factors, may provide greater insight into the sex heterogeneity underlying AD.

LIMITATIONS

The present study is limited by multiple factors. Although the GCTA power was adequate, the exploratory sex-stratified

TABLE 1 | Significant MAGMA-annotated genes (p-value < 2.61×10^{-6}) based on sex-stratified GWAS.

Gene	Chr	p-value	Top SNP in Gene	A1/A2	Male				Female				Heterogeneity between sexes
					Freq	N	OR (95% CI)	p-value	Freq	N	OR (95% CI)	p-value	p-value
Top genes in male													
APOC1	19	1.33×10^{-15}	rs12721051	G/C	0.755	7,374	2.80 (2.70–2.90)	9.71×10^{-97}	0.761	10,968	3.16 (2.37–3.94)	1.89×10^{-182}	0.060
APOE	19	7.49×10^{-15}	rs429358	C/T	0.774	8,060	3.25 (3.15–3.34)	5.04×10^{-122}	0.784	11,793	3.66 (3.57–3.74)	1.78×10^{-215}	0.067
TOMM40	19	1.77×10^{-14}	rs59007384	T/G	0.713	7,699	2.42 (2.33–2.51)	7.85×10^{-85}	0.720	11,249	2.49 (2.42–2.56)	1.20×10^{-138}	0.614
PVRL2	19	2.42×10^{-14}	rs6857	T/C	0.762	7,955	2.81 (2.71–2.90)	4.65×10^{-101}	0.769	11,613	2.97 (2.89–3.05)	1.95×10^{-171}	0.367
BCL3	19	4.30×10^{-8}	rs2965169	C/A	0.610	6,932	0.81 (0.73–0.90)	5.80×10^{-7}	0.606	10,174	0.82 (0.76–0.89)	2.12×10^{-9}	0.832
Top genes in female													
PVRL2	19	1.93×10^{-19}	rs6857	T/C	0.762	7,955	2.81 (2.71–2.90)	4.65×10^{-101}	0.769	11,613	2.97 (2.89–3.05)	1.95×10^{-171}	0.367
APOC1	19	8.88×10^{-16}	rs12721051	G/C	0.755	7,374	2.80 (2.70–2.90)	9.71×10^{-97}	0.761	10,968	3.16 (2.37–3.94)	1.89×10^{-182}	0.060
APOE	19	1.50×10^{-15}	rs429358	C/T	0.774	8,060	3.25 (3.15–3.34)	5.04×10^{-122}	0.784	11,793	3.66 (3.57–3.74)	1.78×10^{-215}	0.067
TOMM40	19	5.00×10^{-10}	rs59007384	T/G	0.713	7,699	2.42 (2.33–2.51)	7.85×10^{-85}	0.720	11,249	2.49 (2.42–2.56)	1.20×10^{-138}	0.614
BCL3	19	1.15×10^{-9}	rs2965169	C/A	0.610	6,932	0.81 (0.73–0.90)	5.80×10^{-7}	0.606	10,174	0.82 (0.76–0.89)	2.12×10^{-9}	0.832
BCAM	19	8.00×10^{-9}	rs28399637	A/G	0.714	5,028	1.52 (1.41–1.62)	1.69×10^{-15}	0.719	7,329	1.54 (1.45- 1.62)	3.71×10^{-25}	0.852

The top SNPs with smallest p-values within genes are shown. Abbreviations: Chr, chromosome; A1, effect allele; A2, non-effect allele; Freq, allele frequency of A1; N, sample size; OR, odds ratio; CI, confidence interval.

GWA studies were underpowered given the available sample size. There was also a sizable difference in sample sizes with a male-to-female ratio of close to 1:1.5, although we verified the results with a matched female sub-cohort to avoid false positive findings simply due to discrepancy in statistical power. In addition, effects of sex chromosomes were not included in this study, which may also be crucial in AD or aging (McCartney et al., 2019).

CONCLUSION

In the present study, we discovered a high genetic correlation of AD between men and women. The overall genetic architecture of AD is similar between sexes, in contrast to genetic heterogeneity across age. Previously reported higher risk from *APOE* ε3/ε4 genotype in females than males among the age group 65–75 years was replicated. Effect sizes of sex-difference variants are likely to be small and large GWAS are needed for discovering such variants. Sex-specific effects from epigenetic variations and gene-environment interactions warrant future investigation to reveal the underlying mechanisms that explain the clinically observed sex differences in AD.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available through the National Institute on Aging Genetics of Alzheimer's Disease Data Storage Site (NIAGADS), NIA's qualified access data repository (https://www.niagads.org/home).

AUTHOR CONTRIBUTIONS

C-HC, HW, and M-TL contributed to the conception and design of the study. HW, M-TL, RS, MF, and C-HC contributed to the data analysis. HW, M-TL, SR, CM, OA, RS, LM, MF, and C-HC contributed to data interpretation. HW, M-TL, LM, MF, and C-HC contributed to drafting the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnagi.2021.674318/full#supplementary-material.

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frontiers in Aging Neuroscience

REVIEW
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Mechanistic Analysis of Age-Related Clinical Manifestations in Down Syndrome

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Down syndrome (DS) is the most common genetic cause of Alzheimer's disease (AD) due to trisomy for all or part of human chromosome 21 (Hsa21). It is also associated with other phenotypes including distinctive facial features, cardiac defects, growth delay, intellectual disability, immune system abnormalities, and hearing loss. All adults with DS demonstrate AD-like brain pathology, including amyloid plaques and neurofibrillary tangles, by age 40 and dementia typically by age 60. There is compelling evidence that increased *APP* gene dose is necessary for AD in DS, and the mechanism for this effect has begun to emerge, implicating the C-terminal APP fragment of 99 amino acid (β-CTF). The products of other triplicated genes on Hsa21 might act to modify the impact of *APP* triplication by altering the overall rate of biological aging. Another important age-related DS phenotype is hearing loss, and while its mechanism is unknown, we describe its characteristics here. Moreover, immune system abnormalities in DS, involving interferon pathway genes and aging, predispose to diverse infections and might modify the severity of COVID-19. All these considerations suggest human trisomy 21 impacts several diseases in an age-dependent manner. Thus, understanding the possible aging-related mechanisms associated with these clinical manifestations of DS will facilitate therapeutic interventions in mid-to-late adulthood, while at the same time shedding light on basic mechanisms of aging.

Keywords: Down syndrome, Alzheimer's disease, hearing loss, infection, COVID-19, mechanisms

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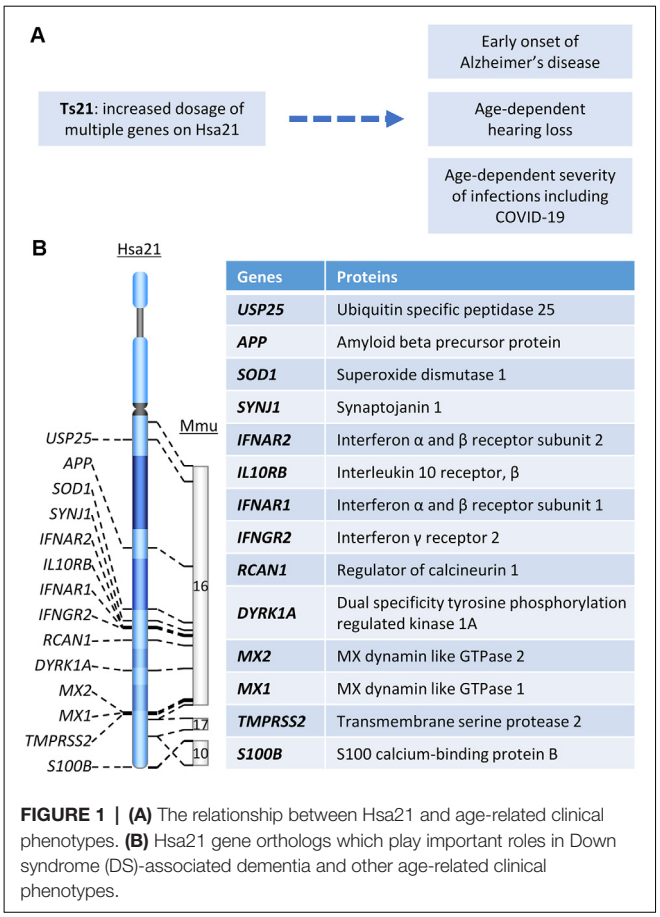
Abbreviations: ABR, auditory brainstem response; ACE2, angiotensin-converting enzyme 2; AD, Alzheimer's disease; AD-DS, Alzheimer's disease in Down syndrome; AICD, APP intracellular domain; APP, Amyloid Precursor Protein; ARDS, acute respiratory distress syndrome; β-CTF, C-terminal APP fragment of 99 amino acids; BFCN, basal forebrain cholinergic neurons; CRP, C-reactive protein; DS, Down syndrome; FAD, familial Alzheimer's disease; Hsa21, human chromosome 21; IFN, interferon; IFNAR, interferon-α/β receptor; IL-10, interleukin-10; ILVs, intraluminal vesicles; IP-10, interferon γ-induced protein 10; iPSC, induced pluripotent stem cells; ISG, interferon-stimulated gene; ISR, integrated stress response; MCP-1, monocyte chemoattractant protein-1; MDA5, melanoma-differentiation-associated protein-5; mTOR, the mammalian target of rapamycin; MVB, multivesicular bodies; NFTs, neurofibrillary tangles; p-tau, phosphorylated tau; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1α; PRR, pathogen recognition receptor; PS1, presenilin-1; RIG-I, retinoic acid-inducible gene 1 protein; SNV, single nucleotide variant; TLRs, toll-like receptors; TMPRSS2, transmembrane protease serine 2; TNF-α, tumor necrosis factor-α; Ts21, trisomy 21; α-CTF, C-terminal APP fragment of 83 amino acids.

INTRODUCTION

Down syndrome (DS), associated with trisomy 21 (Ts21), occurs in 1 in ∼800 live births, leading to an estimated 200,000–250,000 people with this condition in the US (Bull, 2020). It is the most common genetic cause of developmental intellectual disability and early-onset Alzheimer's disease (AD) with very high penetrance (Dierssen, 2012; Strydom et al., 2018; Antonarakis et al., 2020). Children and adults with DS also have an increased incidence of several other important medical conditions that are discussed here. With improved medical care and social support, life expectancy in DS has substantially risen to 60 years, with many living into their 70s (Bittles and Glasson, 2004; Henderson et al., 2007). However, with this increased lifespan, more individuals with DS become affected by age-related clinical phenotypes, a problem that is thought to be aggravated by accelerated biological aging in this syndrome. Thus, research on age-related phenotypes in the DS is becoming increasingly active and important. In this review, we discuss DS-associated AD, age-related hearing loss, bacterial and viral infections including COVID-19, and other age-related medical conditions in DS (**Figure 1A**) that we are investigating in our laboratories.



Genes	Proteins
USP25	Ubiquitin specific peptidase 25
APP	Amyloid beta precursor protein
SOD1	Superoxide dismutase 1
SYNJ1	Synaptojanin 1
IFNAR2	Interferon α and β receptor subunit 2
IL10RB	Interleukin 10 receptor, β
IFNAR1	Interferon α and β receptor subunit 1
IFNGR2	Interferon γ receptor 2
RCAN1	Regulator of calcineurin 1
DYRK1A	Dual specificity tyrosine phosphorylation regulated kinase 1A
MX2	MX dynamin like GTPase 2
MX1	MX dynamin like GTPase 1
TMPRSS2	Transmembrane serine protease 2
S100B	S100 calcium-binding protein B

FIGURE 1 | (A) The relationship between Hsa21 and age-related clinical phenotypes. **(B)** Hsa21 gene orthologs which play important roles in Down syndrome (DS)-associated dementia and other age-related clinical phenotypes.

MECHANISMS OF ALZHEIMER'S DISEASE IN DOWN SYNDROME

Alzheimer's Disease-Related Dementia and Neuropathology in DS

Dementia is defined as a decline in cognitive function sufficient to interfere with a person's ability to conduct a normal daily life. AD is the most common type of dementia with clinical manifestations including memory loss, language problems, cognitive decline, and behavior dysfunction (Scheltens et al., 2016). AD dementia follows a progressive course in which early subtle changes in memory are followed in time by worsening function, leading to the inability to carry out many facets of daily life, with the disintegration of personality (DeTure and Dickson, 2019). DS, the most common genetic cause of AD, is due to trisomy for all or part of chromosome 21 (Hsa21; **Figure 1A**). Due to the increased dosage of genes on Hsa21, DS presents with findings related to dysfunction of multiple body systems. Clinical manifestations apparent even in the newborn period are changes in craniofacial anatomy. Compromised cognition and the delays in development of intellectual and behavioral milestones are essentially universal in children (Antonarakis et al., 2020). By age 40, almost all individuals with DS show AD-like neuropathology and by age 56 fully one-half are diagnosed with dementia (Chen and Mobley, 2019a). The prevalence of dementia in DS was reported to range from 30 to 75%; some studies estimate greater than 80% of dementia beyond age 65 (Zigman et al., 1997; Hithersay et al., 2017). Clinical and neuropathological similarities justify the designation of AD in DS (AD-DS).

The neuropathological changes in AD-DS are much like those in non-DS AD, including amyloid plaques and neurofibrillary tangles (NFTs). Amyloid plaques are extracellular accumulations of amyloid derived from Aβ peptides of various lengths, which are products of Amyloid Precursor Protein (APP) processing (**Figure 1B**, see below; Chen and Mobley, 2019a; Lott and Head, 2019). In AD, however, deposition of Aβ in amyloid plaques routinely fails to show a correlation with dementia, while there is a consistent correlation between disease progression and NFTs. The latter are composed of aberrantly folded and abnormally phosphorylated tau (p-tau; Chen and Mobley, 2019a). A recent study confirms the same pattern for AD-DS. Comparing DS with and without dementia with respect to cortical and striatal plaques and tangles showed that plaques did not predict AD in DS subjects, while abnormal tau aggregation in tangles was correlated with dementia (Perez et al., 2019). The neuropathological features shared between non-DS and DS-associated AD have been recently reviewed (Chen and Mobley, 2019a; Lott and Head, 2019).

Role of *APP* Triplication in AD-DS

Rare cases of early-onset AD are due to duplication of a small *APP* gene-containing chromosomal segment (**Figure 1B**), which is evidence that increased *APP* gene dosage is sufficient to cause AD (Cabrejo et al., 2006; Sleegers et al., 2006). The evidence is likewise compelling that increased *APP* copy number is necessary for AD-DS (Prasher et al., 1998; Doran et al., 2017). Neuropsychological and pathological studies in two partial trisomy DS subjects demonstrated sharing of several typical phenotypic features of DS (short stature, Brushfield spots,

hearing problems, etc.). Though harboring duplicated segments of varying length, in both cases the *APP* gene was present in two, not three, copies. Both died at advanced age free of dementia and the neuropathological hallmarks of AD (Prasher et al., 1998, Doran et al., 2017). These data converge with those in mouse models to demonstrate the necessity of increased *App* gene dose for AD-relevant phenotypes in DS (Salehi et al., 2006, 2009). Although triplication of other genes in Hsa21 has been explored for an effect on AD-linked neuropathologies in DS mouse models, direct evidence linking any other triplicated gene to AD pathology is as yet lacking. Nevertheless, it is likely that other genes will contribute. For instance, DYRK1A (**Figure 1B**) was shown to impact APP processing (Branca et al., 2017) and modify tau phosphorylation (Ryoo et al., 2007). Rcan1 can also modulate tau phosphorylation by both decreasing *p*-tau dephosphorylation and increasing tau phosphorylation (Lloret et al., 2011; **Figure 1B**). Reduction of synaptojanin 1 improved amyloid-induced neuropathology and behavior deficits through accelerating Aβ clearance in one human Swedish APP and FAD (familial AD)-linked PS1 (presenilin-1) double mutant transgenic mouse (Zhu et al., 2013; **Figure 1B**). Moreover, synaptojanin 1 was also linked to enlargement of early endosome in DS (Cossec et al., 2012).

APP is a type 1 transmembrane protein and can be processed by two pathways: the non-amyloidogenic pathway and the amyloidogenic pathway. In the former, APP is sequentially cleaved by α-secretase to produce the soluble fragment sAPPα and α-CTF (C-terminal APP fragment of 83 amino acids); in the latter, APP is cleaved by β-secretase to form sAPPβ and β-CTF (C-terminal fragment of 99 amino acids). α-CTF is then cleaved by γ-secretase to yield the APP intracellular domain (AICD) and the P3 peptide; cleavage of β-CTF yields the same AICD and Aβ peptides of varying length (Zhang et al., 2011; Chen and Mobley, 2019a). Triplication of *APP* gene in DS predicts increased levels in APP and its products (Nistor et al., 2007; Iulita et al., 2014; Chen X. Q. et al., 2021). Consistently, reducing *APP* gene dose to two by CRISPR/Cas9 in DS induced pluripotent stem cells (iPSCs)-derived cortical neurons almost normalized the levels of APP, Aβ42, and the Aβ42/40 ratio (Ovchinnikov et al., 2018). APP and Aβ peptide have been linked to tau pathology in several studies (Hardy and Selkoe, 2002; Kwak et al., 2020), however, this conclusion was contested when normalizing *APP* gene dose in DS iPSC-derived neurons did not impact tau hyperphosphorylation (Ovchinnikov et al., 2018). In contrast, we found that treating with Posiphen to normalize APP levels in the Ts65Dn mouse reduced the levels of not only Aβ42 but also p-tau to levels in the brains of 2N (i.e., euploid) mice (Chen X. Q. et al., 2021; Chen, 2021). Considering that protein products of other triplicated genes on Hsa21, including Dyrk1a and Rcan1, have been shown to contribute to tau hyperphosphorylation in DS (Antonarakis et al., 2020), whether or not and to what extent APP and/or its products are linked to tau pathology need further elucidation. Nevertheless, evidence supporting a role for *APP* gene dose in both Aβ and tau-related pathologies were those for the two partial Ts21. Neither of them showed the senile plaques or NFTs typical of the AD-DS brain (Prasher et al., 1998; Doran et al., 2017).

AD-Associated Features in DS: Endosomal Abnormalities

DS mouse models support research on mechanisms leading to AD-DS (Davisson et al., 1990; Yu et al., 2010a; Herault et al., 2017). Due to *App* gene dosage, APP along with its processing products including CTFs and Aβ peptides is significantly increased in Ts65Dn mice. Normalizing *App* gene copy number in these mice ($Ts65Dn^{APP++-}$) restored the levels of APP and its CTFs (Salehi et al., 2006). It was noted that in $Ts65Dn^{APP++-}$ mouse, reduced NGF transport in basal forebrain cholinergic neurons (BFCNs), as well as the BFCN atrophy, were both significantly improved, pointing to defective retrograde signaling of NGF as contributing to BFCN loss (Salehi et al., 2006). Further studies linked the deficits in NGF axonal transport to abnormal early endosome pathologies including endosome enlargement and Rab5 hyperactivation (Xu et al., 2016).

Apparent enlargement of early endosomes due to excessive activation of small GTPase Rab5 is another shared hallmark of AD in non-DS and DS, one that emerges decades before the appearance of amyloid plaques and NFTs (Cataldo et al., 2000; Chen and Mobley, 2019b). A recent study using ultrastructural methods found that endosomes were clustered in fibroblasts and DS induced pluripotent stem cells (iPSCs)-derived cortical neurons from DS individuals and BFCNs of the Ts65Dn DS mouse model (Botte et al., 2020). They interpreted these findings as evidence that clustering of endosomes is responsible for their apparent enlargement. Whether enlarged or clustered, the significant upregulation in the levels of active Rab5 (GTP-loaded Rab5), which drives endosome fusion, support the importance of changes in early endosomes in DS and AD-DS, as well as in non-DS AD (Xu et al., 2016; Chen X. Q. et al., 2021). Evidence from multiple studies using APP knockdown, the $Ts65Dn^{APP++-}$ mouse, and APP/β-CTF overexpression support that increased *APP* gene dose induces early endosome enlargement and point to β-CTF as the major driver of this change (Salehi et al., 2006; Jiang et al., 2010; Kim et al., 2016; Xu et al., 2016; **Figure 2A**).

Importantly, β-CTF mediated atrophy was prevented by a dominant negative version of Rab5 pointing to an essential role for Rab5 hyperactivation in this process (Xu et al., 2016). The roles of *App* gene dose in abnormal endosome phenotypes and deficient axonal transport of neurotrophin signaling were further supported by a recent study in which Posiphen reversed Rab5 hyperactivation, restored the size of early endosomes, and restored retrograde axonal transport of neurotrophins in primary cortical Ts65Dn neurons and of neurotrophin signaling in the Ts65Dn brains, with the drug acting, at least in part, through reducing the levels of APP and CTFs in a translation-dependent manner (Chen X. Q. et al., 2021). These data are evidence that increased *APP* gene dose in DS, along with increased levels of APP and its products, including β-CTF, acts to induce Rab5 hyperactivation and reduce retrograde transport of neurotrophin signaling, thus linking *APP* gene dose to neurodegeneration.

Early endosomes are upstream of late endosomes and multivesicular bodies (MVB) whose contents are then moved to lysosomes (Grant and Donaldson, 2009). The MVB is a specialized endosome characterized by intraluminal

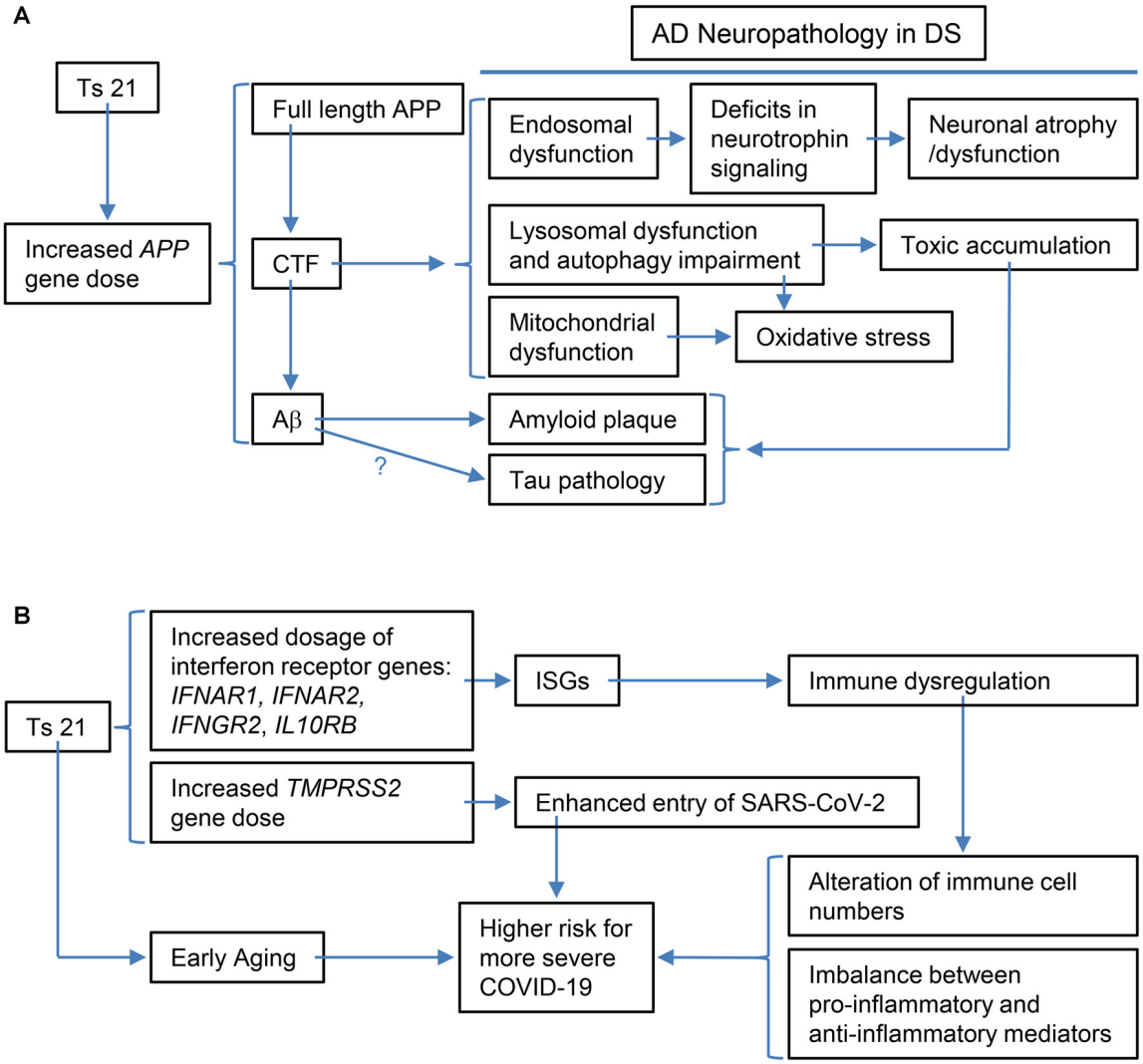


FIGURE 2 | Schematic representations of the specific examples of the relationship between the triplication of Hsa21 or Hsa21 gene ortholog(s) and its phenotypic consequences at various levels. **(A)** The triplication of the *APP* ortholog and its impacts on Alzheimer's disease (AD)-related phenotypic features: increased *APP* gene dose in DS leads to increases in full-length APP protein and its products, including CTF (α-CTF and β-CTF) and Aβ peptides of varying length. Accumulating evidence points to β-CTF as driving endosomal dysfunction, lysosomal dysregulation, autophagy impairment as well as mitochondrial dysfunction. Abnormal early endosomes may contribute to deficits in the retrograde axonal transport of neurotrophic signaling in several neuron populations, including BFCNs, thus compromising their trophic support and leading to neuronal dysfunction and atrophy. Lysosomal dysregulation and autophagy impairment can allow for the build-up of toxic proteins and induce oxidative stress due to failed clearance of organelles, including mitochondria. β-CTF accumulation could also lead to mitochondrial dysfunction. In addition, increased Aβ peptides contribute to amyloid plaque formation; evidence supports a role for Aβ in tau pathology, but as yet there is no direct demonstration for this in DS. Autophagy-lysosomal system dysfunction also contributes to amyloid and tau pathologies. **(B)** The triplication of Hsa21 or Hsa21 gene orthologs and its impacts on the immune system and COVID-19 in the DS population: The dosage increase of the four interferon receptor genes in Ts21, *IFNAR1*, *IFNAR2*, *IFNGR2*, and *IL10RB*, up-regulates expression of interferon-stimulated genes (ISGs), which in turn results in immune dysregulation, including alteration of immune cell numbers as well as an imbalance between pro-inflammatory and anti-inflammatory mediators. These changes in DS are likely related to a higher risk for more severe COVID-19, which may also be contributed by the dosage increase of *TMPRSS2* and early aging. BFCNs, basal forebrain cholinergic neurons; α-CTF, C-terminal APP fragment of 83 amino acids.

vesicles (ILVs) that bud inward into the endosomal lumen. If an MVB fuses with the plasma membrane, the ILVs can be released into the extracellular space as exosomes (Hanson and Cashikar, 2012). Suggested to serve as a buffering mechanism to alleviate endosomal dysfunction, exosome release from MVBs is increased in DS brains and Ts21 fibroblasts as well as in DS mouse models, possibly through the enhanced expression of CD63 which regulates exosome biogenesis (Gauthier et al., 2017). Upregulation of intraluminal vesicles (ILVs) in MVBs was reported in the Ts2Cje model of DS (D'Acunzo et al., 2019). It is noteworthy that exosomes contain APP-derived metabolites raising the possibility of a protective role in removing toxic products, including the C-terminal fragment of 99 amino acids (β-CTFs) and Aβ species

(Perez-Gonzalez et al., 2020). If exosomes play this role, they may enhance Aβ clearance through a microglial cell-dependent pathway. However, as a mediator of intercellular communication exosomes might also propagate and spread Aβ- and tau-related pathologies within neuronal circuits (Mathews and Levy, 2019). Dysregulation of the retromer complex system, which sorts and traffics proteins from endosomes to the trans-Golgi network or the cell surface, was also recently reported to be an early event in the development of AD pathology and cognitive decline in DS (Curtis et al., 2020). Finally, there is evidence pointing to lysosome-autophagy deficits in DS (reviewed in Nixon, 2017; Colacurcio et al., 2018).

In addition to disrupting early endosomes, β-CTF was also shown to mediate APP-induced dysfunction of the lysosomal system through affecting the expression and maturation and/or activity of lysosomal enzymes including cathepsin D, possibly through APP-induced abnormal lysosomal acidification in DS fibroblasts and the Ts2 mouse model (Jiang et al., 2019). These findings were supported in studies in an AD mouse model (3xTgAD) and adeno-associated viral-mediated β-CTF-infected mice (Lauritzen et al., 2016; **Figure 2A**). In the latter, the aggregation of β-CTF in endosomal-autophagic-lysosomal vesicles caused disrupted lysosomal proteolysis and autophagic impairment (Lauritzen et al., 2016).

AD-Associated Features in DS: Mitochondrial Deficits

Mitochondria are membrane-bound organelles that supply ATP as a source of energy for cell function, and mitochondrial dysfunction is another hallmark of DS (Izzo et al., 2018). Several lines of evidence suggest that impaired activity of peroxisome proliferator-activated receptor gamma coactivator 1α (PGC-1α) and hyperactivation of the mammalian target of rapamycin (mTOR) kinase contribute to this dysfunction (Mollo et al., 2020). Mitophagy, which clears damaged mitochondria, was recently demonstrated to be deficient in DS fibroblasts leading to the accumulation of damaged mitochondria. This study showed that reductions in PARKIN and PINK1 impaired initiation of mitophagy, together with hyperactivation of mTOR (Bordi et al., 2019). Consistently, inhibition of mTOR with rapamycin reduced APP levels and attenuated the neurodegenerative phenotypes linked to APP overexpression in Ts65Dn mice (Tramutola et al., 2018). A role of "mitovesicle", a recently defined extracellular vesicle containing mitochondrial components, was suggested as further reflecting mitochondrial pathology in DS (D'Acunzo et al., 2021).

Accumulation of damaged mitochondria is associated with oxidative stress (Bordi et al., 2019), and recent studies linked mitochondrial defects and oxidative stress with insulin resistance in the development of AD pathology in DS (Lanzillotta et al., 2021). Oxidative stress can drive protein oxidation and the formation of protein aggregates (Lanzillotta and Di Domenico, 2021) which are then cleared by protein quality control systems. Among the latter, the integrated stress response (ISR) downregulates protein synthesis to respond to stress. ISR was shown to be activated in the brains of Ts65Dn DS mouse models and DS patients, and suppression of the ISR reversed changes in translation and rescued deficits in synaptic plasticity and long-term memory (Zhu et al., 2019).

A possible link between β-CTF and abnormal mitochondrial structure and function as well as mitophagy defects was supported. The β-CTF was demonstrated to induce mitochondrial morphology alterations and overproduction of mitochondrial reactive oxygen species and to elicit mitophagy failure (Vaillant-Beuchot et al., 2021; **Figure 2A**). Moreover, β-CTF was shown to accumulate in mitochondria-associated membranes and to regulate extracellular cholesterol uptake and trafficking (Montesinos et al., 2020). Though not documented in the context of DS, accumulation of β-CTF has also been linked to inflammation, synaptic dysfunction as well as behavior deficits in the mouse. The unique contribution of β-CTF to AD pathology has been reviewed (Checler et al., 2021). In addition, the contribution of *APP* gene dose-induced increases in Aβ peptides including those toxic Aβ oligomers has been extensively explored and reviewed (Head et al., 2016; Chen and Mobley, 2019a).

AD-Associated Features in DS: Neuroinflammation

In addition, neuroinflammation, involving microglial cells and astrocytes, is presumed to represent a response to Aβ and tau pathologies, with the inflammatory events and the transcriptional pathways thus engaged being viewed as contributing to brain pathology in AD (Wilcock, 2012; Kinney et al., 2018; Nott et al., 2019). A recent neuropathology study characterized an early and evolving neuroinflammatory phenotype across the lifespan in DS, with a higher microglial soma size-to-process length ratio and increased levels of several inflammatory cytokines observed in autopsy brains of children and young adults with DS (Flores-Aguilar et al., 2020). Consistently, in the Dp(16)1Yey mouse model of DS microglia were hyperactivated, with increased pro-inflammatory cytokine levels and altered interferon signaling in the hippocampus, and with decreased spine density and activity of hippocampal neurons and hippocampus-dependent cognitive behavioral deficits (Pinto et al., 2020). Both pharmacological depletion of defective microglia and anti-inflammatory treatment with acetaminophen rescued the deficits in these mice, suggesting a link between aberrant microglia and cognitive dysfunction in Dp(16)1Yey (Pinto et al., 2020). But how such treatments can overcome the impact of important triplicated genes, such as *Dyrk1a*, in the mouse model remains to be revealed, in light of the relationship between *Dyrk1a* and inflammation (Latour et al., 2019).

Unlike non-DS AD, the brain in DS harbors triplication of many inflammation-related genes, including *SOD1*, *S100B*, and genes encoding multiple interferon receptors and several interferon target genes on Hsa21, raising the possibility that DS constitutes a unique environment for the inflammatory signals characteristic of AD neuropathology (**Figure 1B**; Wilcock, 2012). Interestingly, USP25, a deubiquitinating enzyme encoded on Hsa21 (**Figure 1B**), has been linked to aberrant microglia activation as well as deficient synaptic and cognitive function. Both genetic ablation and pharmaceutical inhibition

of USP25 reduced microglia-mediated neuroinflammation and restored synaptic and cognitive function in the 5xFAD mouse model of AD (Zheng et al., 2021).

HEARING LOSS IN DOWN SYNDROME

DS is characterized by a variety of craniofacial anomalies such as softening of the tissue above the larynx (laryngomalacia) and narrowing of the trachea, conditions that contribute to obstructive sleep apnea, voice disorders, and articulatory impairments (Vicente et al., 2020). Individuals with DS tend to have small ears suggestive of the potential for hearing impairments (Aase et al., 1973) The incidence of hearing impairment is relatively high in DS (Keiser et al., 1981; Roizen et al., 1993; Laws and Hall, 2014), with hearing loss ranging from 34% to 78% for children (Shott et al., 2001; Yam et al., 2008; Raut et al., 2011). The nature of this hearing loss and its developmental progression is varied and may depend on the nature of the craniofacial anomalies (Diefendorf et al., 1995).

Conductive Hearing Loss

DS generally impairs the transmission of sound through the external ear and middle ear giving rise to what is referred to as a conductive hearing loss—which is characterized by a loss in hearing sensitivity over a broad range of frequencies, unlike sensorineural hearing loss that preferentially affects the high frequencies. In some cases, the craniofacial disorder involves a malformation of the external ear or substantial narrowing of the ear canal. In severe cases, the ear canal can be occluded (atresia) which attenuates sound transmission to the cochlea where the sensory hair cells are located (Diefendorf et al., 1995). The maximum conductive hearing loss is approximately 40 dB.

The medial end of the ear canal terminates at the tympanic membrane, a thin, translucent membrane that separates the external ear from the middle ear. The middle ear space, which consists of an air-filled cavity in the mastoid bone, is connected to the pharyngeal cavity by the Eustachian tube. The Eustachian tube serves to equalize pressure in the middle ear space with the external pressure in front of the tympanic membrane. The middle ear ossicles composed of three extremely small bones (malleus, incus, and stapes) slightly amplify the sound-induced vibrations relayed from the tympanic membrane to the cochlea. One of the most common problems in DS is Eustachian tube malformation that can result in recurrent middle ear infections that can lead to otitis media with effusion. The fluid in the middle ear greatly attenuates the transmission of sound to the cochlea.

Otoscopy can be used to visualize inflammation of the tympanic membrane, fluid buildup in the middle ear, and rupture of the membrane. Middle ear function can be evaluated with impedance-admittance audiometry which involves inserting a specialized probe into the external ear. The device can detect abnormal middle ear pressure, tympanic membrane rupture, immobility of the tympanic membrane caused by fluid in the middle ear, or hypermobility of the tympanic membrane caused by disarticulation of the middle ear ossicles. Among young DS patients with hearing loss, more than 80% were attributed to conductive hearing loss, mostly due to middle ear effusion (Schwartz and Schwartz, 1978; Austeng et al., 2013). Other factors implicated in conductive loss included immobility or deformities to the middle ear ossicles (Balkany et al., 1979). Histological analysis of temporal bones from DS patients revealed numerous differences in the dimensions of the middle ear space and shortening of the length of the cochlea (Igarashi et al., 1977; Harada and Sando, 1981).

Aging and Sensorineural Hearing Loss

Hearing loss in DS can also result from damage to the sensory and neural structures in the cochlea. Some have reported that < 5% of DS patients suffer from sensorineural hearing loss (De Schrijver et al., 2019) whereas others estimate 53% of the cases are of sensorineural origin (Glovsky, 1966; Brooks et al., 1972). One factor that could contribute to the diversity of results is the age of the subjects. Adults are much less likely than children to develop a middle ear infection but are more likely to have sensorineural hearing loss due to aging (presbyacusis) or other factors (Buchanan, 1990).

Most age-related disorders in DS begin around 40, approximately 20 years earlier than the general population (Martin, 1978; Steingass et al., 2011; Carfi et al., 2014; Glasson et al., 2014). To assess the rate of age-related hearing loss in DS, subjects with conductive or mixed hearing loss were excluded from the analysis (Buchanan, 1990). Sloping, high-frequency sensorineural hearing loss characteristic of age-related hearing loss was evident in young DS subjects. Hearing losses in DS reached 90 dB HL in the 51–60 years age group, compared to 50 dB HL in age-matched controls. Age-related hearing loss occurred 20–30 years earlier in DS than controls, indicating that DS accelerates age-related hearing loss, consistent with other reports (Picciotti et al., 2017).

Histological analysis of temporal bones of elderly DS subjects revealed excessive bone growth and blockade of the canals in the bone through which the peripheral auditory nerve fibers of the spiral ganglion travel out to contact the sensory hair cells in the cochlea (Krmpotic-Nemanic, 1970). Others have used computed tomography to evaluate adult DS subjects, and while no ossicular malformations were detected, vestibular and other inner ear malformations were observed in nearly half of DS subjects (Saliba et al., 2014).

Central Auditory Dysfunction

Hearing problems in DS may also relate to auditory structures in the central nervous system. Brain imaging studies indicate total brain volume is reduced by roughly 20% in DS subjects, with major reductions in the brainstem, hippocampus, temporal lobe, and cerebellum (Fujii et al., 2017; Rodrigues et al., 2019). These changes may be due stunted dendrite growth and atrophy (Becker et al., 1991), changes that could disrupt sound-evoked neural activity in the central auditory pathway. The auditory brainstem response (ABR) is an electrophysiological technique used to assess sound-evoked neural activity in the brainstem. In humans, the ABR waveform consist of five positive and negative peaks, numbered I, II, III, IV and V, occurring 1–7 ms following stimulus onset. Among young adults, the amplitudes of the click-

evoked peaks in the ABR waveform were smaller in DS than controls. Stimulus intensities needed to elicit the ABR responses were higher in DS than in controls, indicative of hearing loss and the slope of the latency vs. intensity function in DS was steeper than in controls consistent with a high-frequency sensorineural hearing loss (Widen et al., 1987). In DS infants 12 months or younger, the absolute latencies of the ABR peaks were shorter than normal and the slope of the latency-intensity functions was steeper than normal (Folsom et al., 1983). Others have reported that the interval between peaks I–II and III–IV were shorter than normal in DS, whereas the IV–V interval was longer than normal (Squires et al., 1980) possible due to a smaller brain size or other brainstem abnormalities.

Animal models can be used to explore the mechanisms underlying conductive, sensorineural, and age-related hearing loss in DS. Most of our efforts and those of others have mainly focused on identifying genomic regions associated with the development of otitis media in DS based on analysis of mouse models triplicated for different Hsa21 orthologous regions (Han et al., 2009; Bhutta et al., 2013; Chen et al., 2013). In Ts65Dn mice, which share many phenotypic characteristics with DS, middle ear effusions were present in nearly 75% of these mice whereas effusions were rare in WT controls. The middle ears in these mice showed varying degrees of inflammation, thickening of the middle ear mucosae, and the presence of goblet cells and pathogenic bacteria (Han et al., 2009).

Additional efforts are underway to model and mechanistically understand the origins of hearing disorders in various DS mouse models. One of the problems associated with many mouse models is that they were developed on background strains that exhibit early-onset age-related hearing loss such as the widely used C57BL strain. This strain carries a single nucleotide variant (SNV) of the cadherin 23 gene ($Cdh23^{c.753A}$) that causes age-related hearing loss in wild-type C57BL mice (Johnson et al., 2017). Therefore, this variant should be removed to investigate age-related hearing loss in DS.

The ABR, widely used to investigate age-related hearing loss in mice (Boettcher, 2002; Konrad-Martin et al., 2012; Johnson et al., 2017; Grose et al., 2019), can also be used to assess auditory function in DS mouse models (Widen et al., 1987; Johnson et al., 2017). Tone burst-evoked ABR provides a useful method for assessing function at different frequencies and estimating the amount of hearing loss at different frequencies. In preliminary studies, we have measured ABR thresholds in a small cohort of DS mice (Yu et al., 2010b) backcrossed for four generations on CBA/J mice a strain that shows little evidence of age-related hearing impairment until extremely late in life so B6-specific age-related $Cdh23^{c.753A}$ allele has been converted to CBA/J-specific $Cdh23^{c.753G}$ (Spongr et al., 1997; Zheng et al., 1999; Han et al., 2016). Our preliminary results from 3-month-old mice revealed slightly elevated (∼25 dB) ABR thresholds and slightly reduced ABR amplitudes in DS mice compared to WT mice. Thresholds in DS mice were elevated over a wide range of frequencies, results indicative of a conductive hearing loss (Bhutta et al., 2013). However, histological results are needed to assess the status of the middle ear and cochlea. To determine if age-related hearing loss is more rapid in DS mice, ABR thresholds in DS and WT will be monitored to determine the rate at which age-related hearing loss develops. Afterward, the cochlea will be evaluated to determine the percentage of missing outer hair cells and inner hair cells. Outer hair cell and inner hair cell losses in DS and WT mice will be compared to determine if age-related sensory cell losses are more severe and develop more rapidly in DS mice compared to WT mice (McFadden et al., 1999; Johnson et al., 2010).

Is Hearing Loss Related to Cognitive Decline and Dementia in DS?

Although hearing loss is mainly considered a sensory disorder, the communication difficulties that it imposes often contribute to social isolation and depression. Recent meta-analyses of human data indicate that age-related hearing loss has a significant association with adverse health outcomes. One of the most unexpected findings was that hearing loss was positively correlated with age-related cognitive decline, cognitive impairment, and dementia and there was a non-significant trend for an association with AD. Vascular disorders, social isolation, or impaired verbal communication were suggested as contributing factors (Lin et al., 2011; Su et al., 2017).

Animal models could provide mechanistic insights on the contribution of hearing loss to cognitive decline, dementia, and AD. Recent animal studies have explored the relationship between noise-induced hearing loss and cognitive impairment using hippocampal-dependent maze learning tasks that assess the acquisition and retention of spatial memory. Hearing loss was associated with a significant decline in the acquisition of spatial memory and deficits in spatial memory retention (memory consolidation; Liu et al., 2016; Park et al., 2016; Manohar et al., 2020). Importantly, these deficits were associated a significant decline in hippocampal neurogenesis (Kraus et al., 2010; Newman et al., 2015; Manohar et al., 2020) and an increase in p-tau protein and lipofuscin in the hippocampus (Park et al., 2018). These results are consistent with the view that hearing loss contributes to cognitive decline, but further studies are needed to assess if hearing loss contributes to the development of dementia and AD.

AGING, IMMUNITY, AND INFECTIONS IN DOWN SYNDROME

DS-Associated Immune Dysregulation: Role of Interferon Pathways

As reviewed by us recently, people with DS are at increased risk of various viral and bacterial infections, while at the same time having a markedly increased susceptibility to autoimmune disorders (Yu et al., 2020). Here we further discuss potential mechanisms of dysregulated immunity in DS, including a role for premature aging, and complete the discussion with some thoughts on relevance to the current COVID-19 pandemic (**Figure 2B**).

The immune system defends against invading pathogens with four main steps: recognize, alert, destroy, and clear (Mueller et al., 2020). The normal immune response to infection comprises two parts: the innate and the adaptive immune systems (Bajaj et al.,

2020). The innate immune system is the first line of defense against invading microorganisms and ultimately regulates the adaptive immune response generated by both T and B cells. Viral infections are first detected in host cells by specific pathogen recognition receptor (PRR) sensor molecules, such as the toll-like receptors (TLRs), the retinoic acid-inducible gene 1 protein (RIG-I), and melanoma-differentiation-associated protein-5 (MDA5), which initiate cascades of signaling events that lead to the expression of immune-regulatory and antiviral genes, such as interferons (IFNs) and their downstream target genes (Yoneyama and Fujita, 2010; Murira and Lamarre, 2016).

Indeed, one of the keys linking between Ts21 and immune dysfunction is IFNs and their receptors. Among six IFN receptors, four are encoded by genes clustered on chromosome 21: *IFNAR1* and *IFNAR2*, coding for type I IFN receptors; *IFNGR2*, coding for type II IFN receptor, and *IL10RB*, coding for the receptor required for type III IFN ligands and cytokines like interleukin-10 (IL-10), IL-22, and IL-26 (**Figures 1B**, **2B**; De Weerd and Nguyen, 2012; Espinosa, 2020). Over-expression of these IFN receptors in individuals with DS leads to hyperactivation of IFN signaling in multiple immune and non-immune cell types and a significantly higher level of key cytokines, including C-reactive protein (CRP), IL-2, IL-6, IL-10, tumor necrosis factor-α (TNF-α), interferon γ-induced protein 10 (IP-10), and monocyte chemoattractant protein-1 (MCP-1; Sullivan et al., 2016, 2017; Araya et al., 2019; Espinosa, 2020). IFNs are essential for antiviral immunity and play a key role in immediate antiviral responses to viral infection in an autocrine and paracrine manner through IFN receptors (IFNARs) signaling and subsequent induction of hundreds of interferon-stimulated genes (ISGs; e.g., *MX1*) to inhibit viral replication and spread (Bajaj et al., 2020; Nikolich-Zugich et al., 2020). IFNs also play an important role in immune modulation and inflammation, as type I IFNs (i.e., IFN-a/b) are often considered double-edged swords as both proinflammatory and anti-inflammatory cytokines. Type I IFNs have been associated with promoting several inflammatory and autoimmune diseases (Hall and Rosen, 2010; Barrett et al., 2020) and have also been successfully used for treatment of inflammatory and autoimmune diseases, such as multiple sclerosis (Kieseier, 2011). Interestingly, several downstream target genes of IFN signaling, including *MX1* and *MX2*, are present on Hsa21 (**Figure 1B**) and the *MX1* gene was found to be over-expressed to high levels in Ts21 fibroblasts when these cells entered replicative senescence (Li et al., 2006), presumably reflecting the known relationship of *MX1* expression and type I IFN signaling to DNA damage and telomere erosion in cell senescence (Frisch and Macfawn, 2020), aggravated by the increased gene dosage of both the upstream and downstream components of the IFN pathway in the cells with Ts21.

IFN signaling hyperactivation in DS subjects caused by overexpression of the interferon receptors and target genes is linked with chronic immune dysregulation, which has been demonstrated by an abundance of evidence and can be characterized by being more predisposed to bacterial infection in the respiratory tracts, weaker response to antibody, and high level of autoantibodies (Ram and Chinen, 2011; Espinosa, 2020; Gensous et al., 2020). Such an immune dysregulation also results in an imbalance between proinflammatory and anti-inflammatory mediators (Hadjadj et al., 2020; **Figure 2B**). At the molecular and cellular levels, dysregulation of immunity in DS is frequently associated with a proinflammatory tendency when compared with the general population, even in the absence of any detectable infection. The tendency is reflected by: (A) changes in the number of different types of immune cells (monocytes, dendritic cells, nature kill cells, neutrophils, and T and B cells); (B) overproduction of proinflammatory cytokines, including TNF-α and IL-6 which, relevant to the discussion below, are key predictors of deteriorating health conditions in COVID-19 (Chen et al., 2020; Hadjadj et al., 2020); (C) functional inhibition of the suppressors, like regulatory T cells (Tregs), a key factor to suppress immune response after clearing of viral pathogens (Cetiner et al., 2010; Araya et al., 2019; Espinosa, 2020; Huls et al., 2021).

Aging and Alteration of the Immune System in DS

The above observation raises the topic of early or accelerated aging in DS. Adults with DS experience certain components of premature aging earlier than the general population, with some typical features including wrinkled skin, gray hair, hearing loss, declining immune function, and increased autoimmune diseases. Using a methodology pioneered by Steve Horvath (Horvath, 2013), DNA methylation patterns can be utilized as an "epigenetic clock" that correlates with chronological age and may reflect underlying biological aging. Both Horvath and his collaborators (Horvath et al., 2015) and our group (Mendioroz et al., 2015; Yu et al., 2020) examined epigenetic aging in human DS, using separate sample sets including blood cells (total leukocytes, and in our second study purified T lymphocytes) and brain tissues. Both datasets reveal rapid aging of CpG methylation patterns during fetal and early postnatal development in DS, leading to a higher "set point" of epigenetic age established by young adulthood, followed by maintenance of this methylation age differential (older in DS than controls), without further acceleration of the difference, throughout adult life (Mendioroz et al., 2015; Yu et al., 2020). Because of the aforementioned association with immune dysregulation and premature aging, it has been proposed that the DS phenotype may include immunosenescence (Gensous et al., 2020).

COVID-19 in DS

COVID-19, caused by the SARS-CoV-2 coronavirus, is currently a major and too often lethal disease worldwide. Since the clinical outcomes of COVID-19 largely depend on the severity of inflammation and differ strikingly by age (more severe in the elderly), studying this disease in DS may prove to be highly informative. The impacts of COVID-19 on individuals with DS were illustrated by a recent international survey initiated by the Trisomy 21 Research Society and the UK ISARIC4C. One thousand forty-six cases of COVID-19 patients with DS from April to October, 2020 were analyzed, and the data were compared with the hospitalized COVID-19 patients with or without DS in the UK ISARIC4C survey. Based on this study,

when compared with non-DS COVID-19 patients, COVID-19 patients with DS exhibited more severe symptoms and were around three times more likely to die. Remarkably, the mortality rates increased rapidly in patients with DS older than 40 years, which resembles the mortality rate for patients without DS at ages over 60. The data in the report indicated that individuals with DS, especially those older than 40 years, are more vulnerable and at higher risk for hospitalization and death due to COVID-19 (Huls et al., 2021).

Several factors discussed in the above sections may contribute to this phenomenon. On the one hand, IFN hyperactivity in DS may present stronger defenses against the virus during the initial stage after exposure to SARS-CoV-2, which may dampen the virus load in the infected site. On the other hand, over-production of the proinflammatory IFNs can overwhelm the system, leading to the so-called cytokine storm and inflammation. Induction of IFNs by coronavirus infection has been proposed as a potential mechanism by which coronavirus establishes persistent infection in cells in the central nervous system (Li et al., 2010; Liu et al., 2011). It remains to be seen, however, whether SARS-CoV-2 establishes persistent infection more readily in individuals with DS as compared to the general populations. Furthermore, several other factors may contribute to this balancing act: (A) Transmembrane protease serine 2 (TMPRSS2) primes the S protein of the SARS-CoV-2 virus to bind to its receptor, angiotensin-converting enzyme 2 (ACE2), during infection (Hoffmann et al., 2020; De Toma and Dierssen, 2021) and *ACE2* is an IFN-stimulated gene (Ziegler et al., 2020). Since the *TMPRSS2* gene is on Hsa21, its triplication leads to its overexpression in DS, which in turn enhances the entry of the coronavirus into host cells (**Figures 1B**, **2B**; De Toma and Dierssen, 2021); (B) SARS-CoV-2 appears to induce low levels of type I and type III IFN responses and elevate IL-6 expression (Blanco-Melo et al., 2020), which might delay local IFN responses and thus enable SARS-CoV-2 to evade recognition and attack by immune cells in order to sustain its replication (Acharya et al., 2020). Recent data demonstrated that, different from mild-to-moderately ill patients, severely ill patients showed a highly impaired type I IFN response and elevated level of TNF-α and IL-6 (Hadjadj et al., 2020); and (C) Aging impaired and delayed the production of type I IFNs (Bajaj et al., 2020) and DS is associated with premature aging, which could be an explanation for initial findings of a higher risk of mortality of DS patients with COVID-19 at ages over 40 years (Huls et al., 2021). On the other hand, as recently proposed, the unusual IFN hyperactivation-related immune dysregulation in DS might in theory also contribute to more frequent cytokine storms induced by SARS-CoV-2 infection in individuals with DS (Bajaj et al., 2020). Cytokine storm is considered as one of the major causes of acute respiratory distress syndrome (ARDS) and multiple organ failure (Ye et al., 2020). Consequently, COVID-19 patients with DS may develop more severe complications and have a higher mortality rate, as illustrated in the aforementioned study (Huls et al., 2021; **Figure 2B**).

In addition, DS is associated with other comorbidities, such as obesity, diabetes, hypotonia, obstructive sleep apnea, craniofacial dysmorphogenesis, congenital heart defects, and gastroesophageal reflux (Antonarakis et al., 2020; Startin et al., 2020; Huls et al., 2021), which may lead to a higher probability of developing more severe symptoms and elevated mortality when infected with SARS-CoV-2 (Espinosa, 2020). Premature aging, discussed in the preceding section, is possibly one of the important risk factors for developing severe COVID-19 cases among DS individuals (Horvath et al., 2015; Hithersay et al., 2019; Gensous et al., 2020; Yu et al., 2020; Chen Y. et al., 2021; Huls et al., 2021). Lastly, medical complications from COVID-19 developed in approximately 60% of patients with DS and increased with age, which include viral pneumonia (36%), acute respiratory distress syndrome (34%), and secondary bacterial pneumonia (17%; Huls et al., 2021). Compared with the general population, pulmonary complications were frequently presented with significantly higher mortality in the DS population (Huls et al., 2021). It is reasonable to link these complications with an increased incidence of respiratory tract infections in DS, which is associated with other DS-related abnormalities, such as aberrant airway anatomy and physiology, hypotonia, aspiration, and dysphagia (Bloemers et al., 2010). Moreover, IFN hyperactivity and elevated IL-10 level may play a role in these complications, particularly in secondary bacterial pneumonia in DS patients (Espinosa, 2020).

Modeling and Analysis of Interplays Between Ts21 and SARS-CoV-2

Understanding of the impacts of various factors on disease processes of COVID-19 patients with DS has just been begun. Additional knowledge will be gained by the generation and analysis of model systems. Because of species-specific differences, mouse ACE2 protein does not serve as a effective receptor for SARS-CoV-2. Transferring a human *ACE2* transgenic allele, such as K18-hACE2, to a mouse model of DS is required. The compound models could be used to ascertain similarities and differences with human COVID-19, and the effects of the DS-mimicking genetic background, which include determination of whether viral replication kinetics are altered in the DS-mimicking genetic background and assessment of age-dependency of COVID-19 disease severity. The models can also be used to understand the impacts of individual genes, such as *TMPRSS2* and *MX1* by normalizing their gene dosages in the compound mutants.

Aging has important effects on various organ systems in mammals, and accelerated aging in DS individuals illustrates such importance, which is reflected in early-onset AD and hearing loss as well as more severe COVID-19 symptoms at younger ages. A better understanding of the mechanisms underlying these phenomena in individuals with DS and in animal models will enhance our abilities to provide more effective interventions to improve the quality of the lives of this special population.

AUTHOR CONTRIBUTIONS

YY and WM conceived the project. All authors contributed to the article and approved the submitted version.

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REVIEW
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Basal Forebrain Cholinergic Neurons: Linking Down Syndrome and Alzheimer's Disease

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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 understudied 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.

Keywords: basal forebrain cholinergic neurons, down syndrome, Alzheimer's disease, pluripotent stem cell, neurodegeneration

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INTRODUCTION

Down syndrome (DS, trisomy 21, T21) is a complex developmental disorder that arises from trisomy of human chromosome 21 (Hsa21) (Lejeune et al., 1959) and is both a neurodevelopmental and a neurodegenerative disorder. Intellectual disability in individuals with DS ranges from mild to moderate with deficits in specific domains, including attention and memory. DS features arise as a result of uncharacteristic dosage of coding and non-coding sequences found on Hsa21. Despite

its known cause and high incidence (Shin et al., 2009; Presson et al., 2013; de Graaf et al., 2015), little is known about the underlying developmental defects and degenerative outcomes that cause the characteristics of DS.

Down syndrome is also characterized by Alzheimer's disease (AD) pathology that emerges in middle age (Scott et al., 1983; Coyle et al., 1986; Visser et al., 1997; Burt et al., 1998; Menendez, 2005; Head et al., 2012; 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 59 years, 18% between 50 and 54 years, and 32% between 55 and 59 years with a cumulative risk of 90% by age 65 (Zigman et al., 1996; Holland et al., 2000; Head et al., 2016; Sinai et al., 2018). In addition, the prevalence of symptomatic AD in individuals with DS reaches 90–100% by age 70, while only 11.3% of the general population have AD by the age of 65 (Fortea et al., 2020; Alzheimer's Association, 2021). Additionally, there are sex differences, with DS males developing AD-like pathology at an earlier age than females (Zigman et al., 1996; Holland et al., 2000; Head et al., 2012). 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 AD (Yates et al., 1980; Beyreuther and Masters, 1995; Salehi et al., 2004; Baker-Nigh et al., 2015; Chen et al., 2018). 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 (Mesulam et al., 1983a; Woolf, 1991; Ballinger et al., 2016). BFCNs degenerate during aging and cell loss correlates with memory loss in old age and in 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

Basal forebrain cholinergic neurons are a cluster of large neurons in the basal forebrain first described by 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 >100 m (Wu et al., 2014). BFCNs express several neurotransmitter receptors that include adrenergic, glutamatergic, GABAergic, estrogen receptors, and endocannabinoids (Miettinen et al., 2002; Harkany et al., 2003; Mufson et al., 2003; Zaborszky et al., 2004; De Souza Silva et al., 2006). 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 through the lifespan (Sarter et al., 2003; Hasselmo, 2006; Botly and De Rosa, 2009; Heys et al., 2010; Mitsushima et al., 2013; Schmitz and Duncan, 2018).

Basal forebrain cholinergic neurons are classified based on their projection targets defined in rats and non-human primates (Mesulam et al., 1983a,b; Butcher and Semba, 1989; Coppola and Disney, 2018; Solari and Hangya, 2018). 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

Basal forebrain cholinergic neuron 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 (Détári, 2000; Conner et al., 2003; Blanco-Centurion et al., 2007; Weinberger, 2007; Jones, 2008; Kaur et al., 2008; Lin and Nicolelis, 2008; Parikh and Sarter, 2008; Goard and Dan, 2009). 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.

Basal forebrain cholinergic neurons 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 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 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 (Naumann et al., 2002; Mufson et al., 1996, 2003, 2004; Ginsberg et al., 2006). The loss of BFCNs during normal aging and in the pathology of AD highlight 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 unaffected controls suggests that loss of BFCNs contributes to memory loss, decreased spatial recognition and disturbance in language that are common areas of decline in both DS and AD (Davies and Maloney, 1976; Whitehouse et al., 1982; Coyle et al., 1983, 1986; Casanova et al., 1985; Price et al., 1986; Mufson et al., 1989, 1995; Perry et al., 1992; Bierer et al., 1995; Ballinger et al., 2016). 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 (**Figure 1**). Yet, gaps in our understanding of their role specifically in BFCNs remain.

Cholinergic Hypothesis

Because the cholinergic system is important in various forms of dementia, including AD (Davies and Maloney, 1976; Whitehouse et al., 1982; Price et al., 1986; Mufson et al., 1989; Perry et al., 1992; Bierer et al., 1995; Mufson et al., 1995; Ballinger et al., 2016), 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 (Aquilonius et al., 1975; Yates et al., 1980; Arendt et al., 1983; Dubois et al., 1983; Barron et al., 1987; Ferrante et al., 1987; Kato, 1989). 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, 2002; Selkoe and Hardy, 2016). These data and others led to the "cholinergic hypothesis of AD" (Coyle et al., 1983; Contestabile, 2011; 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 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 (Rovelet-Lecrux et al., 2006, 2007; Sleegers et al., 2006; Kasuga et al., 2009; Thonberg et al., 2011; Hooli et al., 2012; McNaughton et al., 2012; Swaminathan et al., 2012; Wiseman et al., 2015). 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

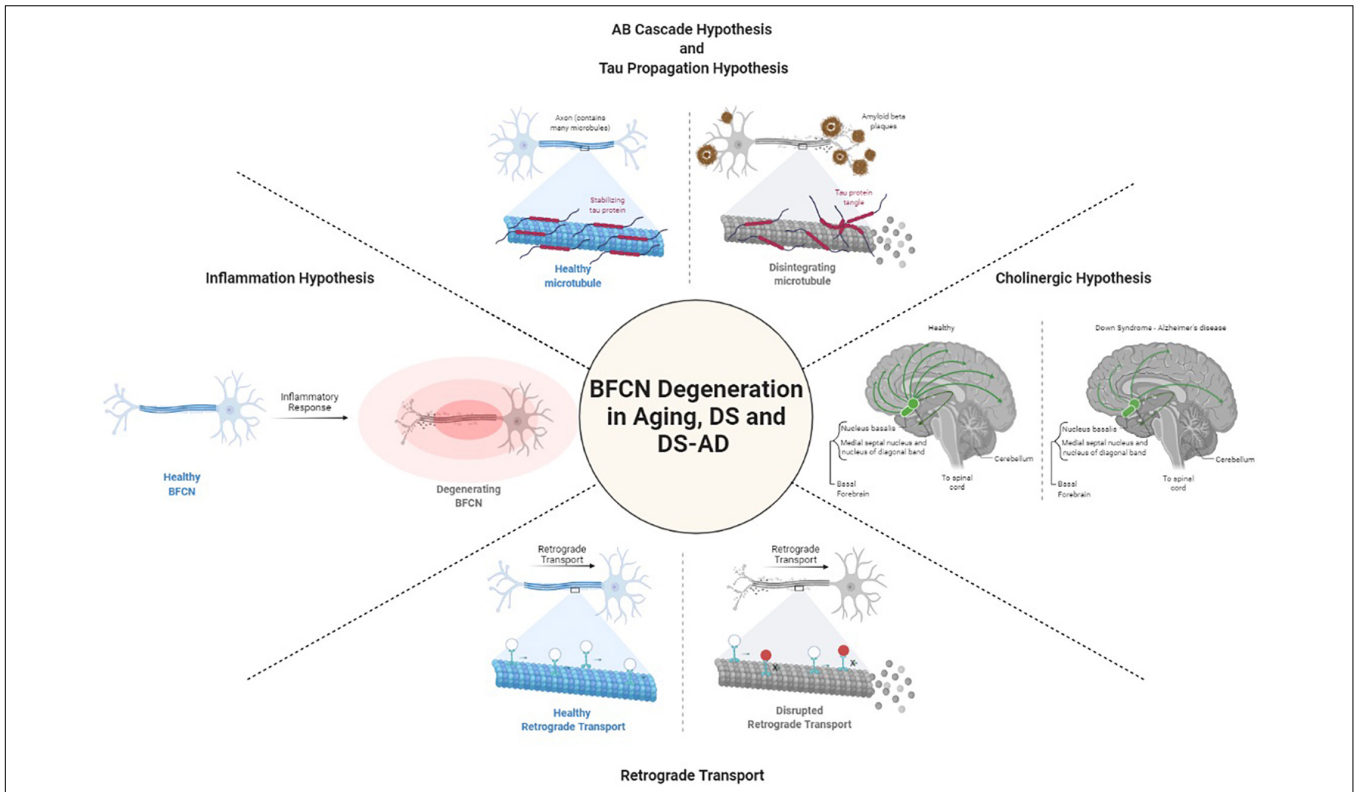

FIGURE 1 | Postulated mechanisms of BFCN degeneration. Created with BioRender.

AD pathogenesis (Wiseman et al., 2015, 2018; Ovchinnikov et al., 2018). 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 (Clavaguera et al., 2009; Frost 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 (Grundke-Iqbal et al., 1986; Kosik et al., 1986; Nukina and Ihara, 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 (Lott and Head, 2019; Perez et al., 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., 2010, 2018). A biomarker study revealed that individuals with DS had decreased amyloid-β over time, while the plasma level of tau and NFT increased leading to a reduction in basal forebrain volume (Mengel et al., 2020; Schmitz 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 two to five-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 (Heneka et al., 2013; Sheedy 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

Basal forebrain cholinergic neurons 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 increase 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 of these 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 T21 cells may also be important in BFCN pathology. For example, oxidative stress 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 human T21 cortical neurons has recently emerged (Botté et al., 2020) and so 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 reflect the local neural vulnerability that spreads overtime (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; Khan et al., 2014; Baker-Nigh et al., 2015; Grothe et al., 2018; Sepulcre et al., 2018; Hanseeuw et al., 2019). Yet, recent imaging studies have started to challenge this model. A longitudinal study using cerebrospinal fluid (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 et al., 2018; Fortea et al., 2020; Rafii 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 (Klunk et al., 2007; Remes et al., 2008; Villemagne et al., 2009; Bateman et al., 2012). 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, 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 (Alzheimer's Association, 2019). Donepezil, galantamine, and rivastigmine are acetylcholinesterase inhibitors, while memantine is a non-competitive 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. Most, if not all, have proven clinically unsuccessful, with Donepezil being the last FDA approved AD drug in 2010. It is important to note that most of the AD drugs are acetylcholinesterase inhibitors that reduce the breakdown of acetylcholine released from BFCNs. Much like donepezil, galantamine works by inhibiting acetylcholinesterase in a reversible and selective manner 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 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, it is clear that 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; Cacabelos, 2007; Folch et al., 2018; 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 in particular 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 *Caenorhabditis elegans* models of PD, treatment of DS induced pluripotent stem cell (iPSC)-derived 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 (Hamlett et al., 2016; Herault et al., 2017). Of the 225 protein coding genes found on Hsa21, 166 are conserved in three regions in mice, murine (mmu) chromosome 10, 16, and 17 (Hattori et al., 2000; Akeson et al., 2001). Mouse models of DS have provided evidence of the influence of individual genes on Hsa21 that lead to deficits in BFCNs (Kiss et al., 1989; Sweeney et al., 1989; Coyle et al., 1991; Cooper et al., 2001; Hunter et al., 2004; Salehi et al., 2006; Ash et al., 2014; Kelley et al., 2014a, 2019; Powers et al., 2016, 2017).

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 1990s 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 (Li et al., 2007; Yu et al., 2010). 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).

Basal forebrain cholinergic neuron 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 (Contestabile et al., 2006; Hamlett et al., 2016). 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 (prior to 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), highlight the importance of cholinergic levels in DS for healthy neural circuits (Alldred et al., 2018, 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 (Thomson et al., 1998; Takahashi et al., 2007; 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; Chou et al., 2012; MacLean et al., 2012; Mou et al., 2012; Briggs et al., 2013; Jiang et al., 2013; Lu et al., 2013; Weick et al., 2013; Chen et al., 2014; Hibaoui et al., 2014; Pipino et al., 2014; Huo et al., 2018). iPSCs are thus a useful model system to study DS and AD (Li et al., 2012; Murray et al., 2015; Ovchinnikov et al., 2018; Real et al., 2018).

Isogenic control iPSCs are important research tools to distinguish the consequences of T21 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, 2–4% of DS cases are mosaic individuals in which their somatic cells are mosaic for T21 (Papavassiliou et al., 2009; Murray et al., 2015). By taking advantage of cellular mosaicis, isogenic T21 and euploid iPSCs can be derived from the same individual (Weick et al., 2013; Murray et al., 2015; Gough et al., 2020).

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 (Park et al., 2008a). Full chromosomal correction of the gene dose imbalance has been accomplished using XIST-mediated (Jiang et al., 2013) or TKNEO-mediated silencing of the trisomic chromosome in iPSCs (Li et al., 2012) and ZSCAN-induced elimination of the extra chromosome (Amano et al., 2015). These strategies may enable 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 (Weick et al., 2013; Huo et al., 2018; Real et al., 2018). T21 iPSC-derived cortical neurons showed impairment in synaptic activity, as well as compensatory responses to oxidative stress (Shi et al., 2012; Briggs et al., 2013; Weick et al., 2013; Sobol et al., 2019).

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 (Xu et al., 2005; Gulacsi and Anderson, 2006; Li et al., 2009). MGE progenitors express the transcription factor NKX2.1, whose expression is regulated by SHH (Du et al., 2008; Xu et al., 2008, 2010). Despite the known development of BFCNs from the MGE, few differentiation protocols have been established to generate BFCNs from hPSCs

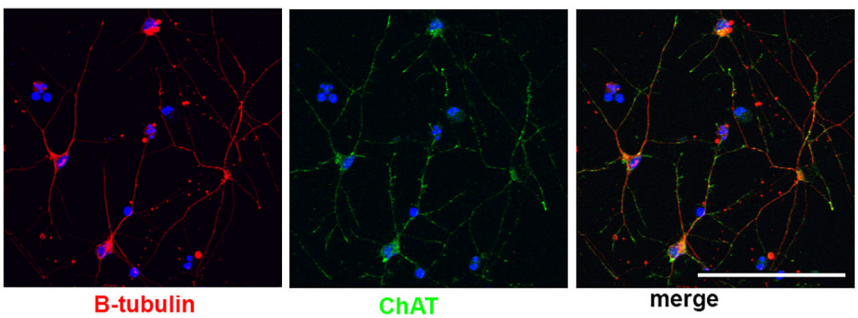

FIGURE 2 | Human stem cell derived BFCNs. Immunofluorescence of BFCNs derived from human iPSCs showing neurons (β-tubulin, red), choline acetyltransferase (ChAT, green), and merged image showing co-expression. Scale bar, 100 um.

(Bissonnette et al., 2011; Liu et al., 2013a; Duan et al., 2014; Hu et al., 2016), and many result in mixed populations of cells. The most robust technique (Liu et al., 2013a; Hu et al., 2016) 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 (Wang and Liu, 2001; Furusho et al., 2006). Approximately 40% of the resulting neurons express ChAT, the enzyme responsible for biosynthesis of the neurotransmitter acetylcholine and a mature BFCN marker (Hu et al., 2016; **Figure 2**). Others have also successfully used small molecules to pattern BFCNs resulting in efficiencies ranging from 15 up to 80% (Liu et al., 2013b; Yue et al., 2015; 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 indicates 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

With an increased life expectancy of DS individuals, it is important to study the cellular and molecular mechanisms that underlie neurodegeneration and AD in DS (Sawada et al., 2008; Duncan, 2011; Baker and Petersen, 2018). 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 BFCNs degeneration may be one of the earliest events in AD and DS neuropathology.

AUTHOR CONTRIBUTIONS

AB and JM contributed to concept and approach of the review. JM collected and organized the research articles and wrote the first draft of the manuscript. MZ and NW wrote and edited sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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MINI REVIEW
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Nerve Growth Factor Compromise in Down Syndrome

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The basal forebrain cholinergic system relies on trophic support by nerve growth factor (NGF) to maintain its phenotype and function. In Alzheimer's disease (AD), basal forebrain cholinergic neurons (BFCNs) undergo progressive atrophy, suggesting a deficit in NGF trophic support. Within the central nervous system, NGF maturation and degradation are tightly regulated by an activity-dependent metabolic cascade. Here, we present a brief overview of the characteristics of Alzheimer's pathology in Down syndrome (DS) with an emphasis on this NGF metabolic pathway's disruption during the evolving Alzheimer's pathology. Such NGF dysmetabolism is well-established in Alzheimer's brains with advanced pathology and has been observed in mild cognitive impairment (MCI) and non-demented individuals with elevated brain amyloid levels. As individuals with DS inexorably develop AD, we then review findings that support the existence of a similar NGF dysmetabolism in DS coinciding with atrophy of the basal forebrain cholinergic system. Lastly, we discuss the potential of NGF-related biomarkers as indicators of an evolving Alzheimer's pathology in DS.

Keywords: Alzheimer's, Down syndrome, nerve growth factor, metabolic pathway, basal forebrain cholinergic neuron, cholinergic dysfunction, neuroinflammation

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INTRODUCTION

Down syndrome (DS), also known as trisomy 21, is a genetic disorder caused primarily by the triplication of chromosome 21, which leads to several abnormalities and lifelong intellectual disability. As DS individuals age, they become at a very high risk of developing Alzheimer's disease (AD). Indeed, DS is now recognized as the most common form of genetic AD, and AD presentation in DS (DSAD) is similar to that of autosomal-dominant AD (ADAD) (Lott and Lai, 1982; Zigman and Lott, 2007; Davidson et al., 2018; Strydom et al., 2018). Therefore, individuals with DS will inevitably develop full-blown AD pathology with extracellular amyloid plaques, intracellular neurofibrillary tangles, neuroinflammation, cholinergic depletion and cognitive and learning deficits leading to clinical dementia in 70% of DS people over 60 years of age (McCarron et al., 2014).

ALZHEIMER PATHOLOGY IN DS

Amyloid and Tau Pathologies

Due in part to the triplication of genes encoding amyloid precursor protein (APP) and β-amyloid cleavage enzyme 2 (BACE2) (St George-Hyslop et al., 1987; Acquati et al., 2000), located on chromosome 21, individuals with DS display a progressive accumulation of amyloid-beta (Aβ) peptides starting before birth (Lemere et al., 1996; Teller et al., 1996; Mori et al., 2002; Iulita et al., 2014). As in ADAD, the AD pathology in DS (DSAD) follows a predictable disease trajectory (Wiseman et al., 2015; Carmona-Iragui et al., 2017). As early as childhood, a fraction of people with DS present diffuse Aβ plaques within their brain (Lemere et al., 1996; Leverenz and Raskind, 1998). Early Tau pathology (detected as AT8 immunoreactivity) in DS appears by middle age (30–40 years) (Head et al., 2003; Davidson et al., 2018), after Aβ pathology is established, and follows a distribution pattern resembling that of AD, starting in the entorhinal cortex and spreading to the hippocampus and the neocortex (Davidson et al., 2018). By 40 years old nearly all DS brains show advanced AD pathology with extensive amyloid plaques and neurofibrillary tangles (NFTs) (Mann, 1988; Lemere et al., 1996; Leverenz and Raskind, 1998; Lott and Head, 2001; Mori et al., 2002; Head et al., 2003). However, DS brains with AD pathology present a higher density of NFTs than that seen in sporadic AD (Hof et al., 1995). A contributing factor may be the triplication of the dual-specificity tyrosine phosphorylated and regulated kinase 1A gene (DYRK1A), also located on chromosome 21, which is known to phosphorylate Tau at several sites relevant to AD (Woods et al., 2001; Liu et al., 2008). The triplication of APP, PS1 and several immune response mediators associated with AD may also play a role (Arron et al., 2006; Ryoo et al., 2008; Ryu et al., 2010; Kurabayashi et al., 2015; García-Cerro et al., 2017). As in sporadic AD, Aβ seems to be the main driver of dementia in DS as indicated by case studies reporting on individuals with DS who had partial trisomy 21 but were disomic for APP and who did not develop plaques, NFTs or dementia (Prasher et al., 1998; Doran et al., 2017). However, as in AD, cognitive decline in DS shows a stronger association with NFTs than with Aβ plaques (Margallo-Lana et al., 2007). Recently, a comprehensive revision of the order and changes in AD biomarkers in adults with DS has been communicated by Fortea and collaborators (Fortea et al., 2020).

It is noteworthy that the presence of the apolipoprotein E ε4 allele (APOEε4), the highest genetic risk factor associated with AD in the general population, is also a major determinant of AD pathogenesis and progression in people with DS. It has been shown that APOEε4 raises the risk for both early-onset and sporadic AD (Corder et al., 1993; Strittmatter et al., 1993; Qian et al., 2017) and accelerates both symptom onset and pathology severity in a gene-dose-dependent manner (Blacker et al., 1997; Farrer et al., 1997; Fleisher et al., 2013; Liu et al., 2013; Gonneaud et al., 2016; Lautner et al., 2017; Cacciaglia et al., 2018; Mishra et al., 2018). Accordingly, 65–80% of all AD sufferers harbor at least one APOEε4 allele (Farrer et al., 1997). The elevated risk of developing dementia conferred by APOEε4 involves mechanisms associated with both Aβ and tau aggregation (Therriault et al., 2020). APOEε4 carriers also have increased blood-brain barrier breakdown that has been shown to predict cognitive decline (Bell et al., 2012; Zhao et al., 2015; Montagne et al., 2020). Similarly, in people with DS the presence of the APOEε4 allele increases the risk of dementia, although to a lesser extent than in the general population (Prasher et al., 2008; Rohn et al., 2014). It also lowers the age of disease onset (Schupf et al., 1996; Deb et al., 2000; Coppus et al., 2008; Bejanin et al., 2021), aggravates Aβ deposition (Hyman et al., 1995; Bejanin et al., 2021), and accelerates neurodegeneration (Bejanin et al., 2021). Additionally, DS individuals harboring the APOEε4 allele are at additional increased risk for early mortality (Prasher et al., 2008; Hithersay et al., 2019).

Neuroinflammation

Neuroinflammation is another paramount feature of AD pathology that contributes to the progression and severity of the disease (Akiyama et al., 2000). The interest in the role of immune processes in AD pathogenesis began with the discovery of major histocompatibility molecules and complement system proteins in amyloid plaques (Jonker et al., 1982), and the description of HLA-DR- and IL-1β-positive reactive microglia surrounding amyloid plaques and neurofibrillary tangles (McGeer et al., 1987, 1988). This concept was reinforced by genome-wide association studies indicating that immune-related genes, such as TREM2, HLA-DRB5-HLA-DRB1, CR1 and CLU are risk factors for AD (Harold et al., 2009; Lambert et al., 2009, 2013; Brouwers et al., 2012; Jonsson et al., 2013). DS brains display lifelong neuroinflammatory changes starting at the fetal stage, prior to plaque deposition. Still, the precise cause of neuroinflammation initiation –triggered either by the accumulating AD pathology or by the triplication of immune-related genes [reviewed in Wilcock (2012)]—remains unclear. Early reports on neuroinflammation in DS described a pronounced proliferation of activated glia overexpressing S100B, another chromosome 21 gene product, and interleukin-1 (IL-1) α and β (Griffin et al., 1989; Royston et al., 1999). Since then, the evolving neuroinflammatory phenotype of DS, which presents both similarities and differences compared to that in sporadic AD, has been increasingly described (Stoltzner et al., 2000; Head et al., 2003; Xue and Streit, 2011; Wilcock et al., 2015; Flores-Aguilar et al., 2020). In fetuses and neonates with DS, neuroinflammation is characterized by an increase in the number of IL-1β-expressing microglia (Griffin et al., 1989). This neuroinflammation escalates as children and young adults with DS show an exacerbated neuroinflammatory profile with activation of the complement pathway, elevated levels of key inflammatory cytokines and altered microglia morphology indicative of activation, including the presence of rod-like microglia (Stoltzner et al., 2000; Wilcock et al., 2015; Flores-Aguilar et al., 2020). Older DS individuals (over 40 years of age) also display increased levels of potent inflammatory cytokines compared to karyotypical controls, although to a lesser extent than their younger DS counterparts. However, an increase of dystrophic microglia with age has been reliably demonstrated (Stoltzner et al., 2000; Wilcock et al., 2015; Flores-Aguilar et al., 2020). Accordingly, elevated cytokine expression and immune dysregulation have been reported in the blood of children and adults with DS (Licastro et al., 2005;

Iulita et al., 2016; Sullivan et al., 2017; Waugh et al., 2019; Weber et al., 2020). It has been proposed that such changes promote AD pathology in DS (Wilcock and Griffin, 2013). Such changes may also be used to predict and monitor pathological progression. For example, longitudinal changes in TNFα, IL-8, and AD biomarkers in plasma along with a nerve growth factor (NGF) metabolism dysregulation could predict prospective cognitive decline in a population of DS individuals asymptomatic for AD (Iulita and Cuello, 2016).

Cholinergic Dysfunction

The cholinergic neurotransmitter system is crucial for cortical and hippocampal activity, learning and memory. Its atrophy and degeneration are central to AD symptomatogenesis (Bowen et al., 1976; Davies and Maloney, 1976; Whitehouse et al., 1981, 1982; Mufson et al., 1989; Grothe et al., 2010; Kerbler et al., 2015). Its role in the AD pathology is highlighted by the fact that four of the five drugs currently approved for AD treatment are acetylcholinesterase (AChE) inhibitors, which, by preventing the breakdown of acetylcholine, increase the cholinergic tone resulting in improved cognitive outcomes, as long as sufficient cholinergic terminals persist in the telencephalon (Hampel et al., 2018; Kabir et al., 2019; Marucci et al., 2020). Degeneration of basal forebrain cholinergic neurons (BFCNs) parallels the development of AD pathology, progressing silently for several years prior to the onset of cognitive symptoms (Grothe et al., 2014), as reviewed by Hampel et al., 2018. Further, the degeneration of BFCNs predicts atrophy of the brain regions innervated by their projections such as the entorhinal cortex and cerebral cortex (Schmitz and Spreng, 2016; Schmitz et al., 2018). Loss of cholinergic innervation has also been linked to vascular dysfunction, another early predictor of the progression to AD (Iturria-Medina et al., 2016), and increased blood-brain barrier permeability (Domer et al., 1983; Radu et al., 2017; Nizari et al., 2019, 2021).

Cholinergic dysfunction in DS was first evidenced by a significant reduction in choline acetyltransferase (ChAT) and AChE activity in the temporal cortex of older individuals with DS, which was not present in a younger DS subject (Yates et al., 1980, 1983). Soon after, a significant and seemingly age-related reduction in volume of the nucleus basalis was also observed (Casanova et al., 1985). Further studies demonstrated that abnormalities in the cholinergic system develop as the individuals age and accumulate AD pathology since fetuses display a neuronal density and vesicular acetylcholine transporter (VAChT) immunoreactivity comparable to controls and that newborns with DS have ChAT activity levels similar to age-matched controls (Kish et al., 1989; Lubec et al., 2001). Age-related atrophy and neurodegeneration of BFCNs is recapitulated in mouse models of DS (Holtzman et al., 1992, 1996; Fiedler et al., 1994; Cooper et al., 2001; Granholm et al., 2002) and was attributed to APP gene triplication through disruption of endosomal phenotype and function (Cataldo et al., 2003). Such cholinergic dysfunction is sex-dependent and can be restored by estrogen treatment (Granholm et al., 2002; Kelley et al., 2014b).

Interestingly, in the Ts65Dn mouse model of DS, maternal supplementation with choline, a critical substrate for the synthesis of acetylcholine, during pregnancy and lactation reduced cognitive dysfunction and degeneration of BFCNs in their adult offspring (Moon et al., 2010; Ash et al., 2014; Kelley et al., 2014a; Strupp et al., 2016; Kelley et al., 2016; Powers et al., 2017). Although the exact mechanisms underlying the effects of choline therapy remain obscure, it has been shown that choline treatment rescued the expression of genes related to the cytoskeleton and cholinergic neurotransmission amongst others (Kelley et al., 2019).

NERVE GROWTH FACTOR METABOLIC DYSREGULATION IN DS

Basal forebrain cholinergic neurons depend on the continuous supply of NGF for the maintenance of their functional phenotype, their synaptic integrity and ultimately their survival (Hefti and Will, 1987; Cuello, 1996; Levi-Montalcini et al., 1996). In the adult CNS it has been demonstrated experimentally that the levels of *endogenous* NGF regulates the day-to-day number of cortical cholinergic synapses (Debeir et al., 1999). These findings led to Appel's hypothesis that the trophic support to BFCNs is compromised in AD (Appel, 1981). However, the levels of NGF transcripts are unaffected (Goedert et al., 1986; Jette et al., 1994; Fahnestock et al., 1996) and the protein levels of the NGF precursor, proNGF, are greatly elevated in AD post-mortem brain samples (Fahnestock et al., 1996, 2004; Peng et al., 2004; Pedraza et al., 2005; Al-Shawi et al., 2008; Bruno et al., 2009a). A resolution of such an apparent paradox and insight into the cause of the cholinergic deficits characteristic of AD was brought about by the discovery of an NGF metabolic pathway controlling the availability of mature NGF (mNGF) as well as its extracellular degradation (Bruno and Cuello, 2006). The pharmacological manipulation of this NGF metabolic pathway has shown it to regulate the cholinergic phenotype of both the cortical synapses and the BFCN cell bodies (Allard et al., 2012, 2018).

In brief, proNGF is released into the extracellular space in response to neuronal or neurotransmitter stimulation. In *ex vivo* studies it has been shown that proNGF (and not mature NGF, mNGF) is released along with a set of zymogens and convertases responsible for its maturation and degradation (Bruno and Cuello, 2006). Maturation of proNGF into mNGF is accomplished by the enzyme plasmin, which is generated by the cleavage of its inactive zymogen, plasminogen, by tissue plasminogen activator (tPA), a process regulated by the tPA inhibitor, neuroserpin (Bruno and Cuello, 2006). Degradation of receptor-unbound mNGF is performed by the matrix metalloproteinases 9 and 3 (MMP-9 and MMP-3), derived from cleavage of their protein precursors, a process regulated by tissue inhibitor of metalloproteinases-1 (TIMP-1) (**Figure 1**; Bruno and Cuello, 2006; Pentz et al., 2021b).

Investigations in post-mortem brain tissue, plasma and cerebrospinal fluid (CSF) revealed that NGF metabolic dysfunction is present in the preclinical and clinical continuum of sporadic AD (Peng et al., 2004; Bruno et al., 2009a,b; Mufson et al., 2012; Hanzel et al., 2014; Pentz et al., 2020). Specifically, both NGF maturation and degradation are

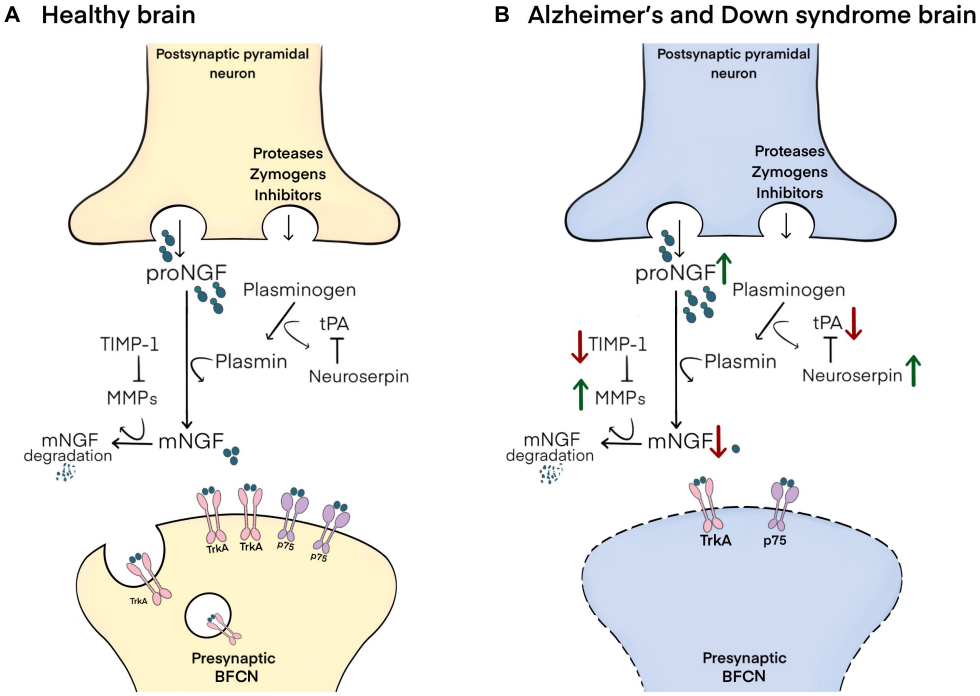

FIGURE 1 | Schematic representation of the NGF metabolic pathway and its altered state in Alzheimer's and Down syndrome pathology. **(A)** In the healthy brain, proNGF is co-released in the extracellular space with zymogens and convertases involved in its maturation and degradation. proNGF is cleaved to yield mNGF by plasmin, itself derived from the cleavage of plasminogen by tPA, a process regulated by neuroserpin. mNGF then dimerizes and binds to p75/TrkA receptor complexes on presynaptic terminals of BFCNs, followed by the retrograde transport of mNGF to their cell bodies in the basal forebrain. Receptor-unbound mNGF is rapidly degraded by MMP-9 and MMP-3, which are produced from their pro-proteins under the control of TIMP-1. **(B)** In Alzheimer's disease and in Down syndrome brains, increased neuroserpin and decreased tPA lead to reductions in the maturation of proNGF to mNGF by limiting plasmin concentrations. Further, decreased TIMP-1 and increased MMP-3/MMP-9 result in the excessive degradation of unbound mNGF. These changes result in impaired trophic support to BFCNs, leading to their atrophy.

disrupted at preclinical AD stages as revealed in individuals with no cognitive impairment (NCI) but with high brain β-amyloid (Aβ) levels (HA-NCI). This NGF dysmetabolim correlated with cerebral Aβ and Tau deposition, cognitive performance, and loss of cholinergic synapses (Pentz et al., 2020). NGF dysmetabolism is also found in the brain of people with prodromal AD, also referred to as mild cognitive impairment (MCI), and with clinical AD as represented by increased levels of proNGF, neuroserpin, as well as MMP-3 and MMP-9 activity (Peng et al., 2004; Bruno et al., 2009a,b; Mufson et al., 2012; Pentz et al., 2020). These findings are also in accordance with other accounts of increased proNGF in CSF from people with AD (E Counts et al., 2016), and with the altered expression of MMP-3, neuroserpin, and plasminogen reported in CSF from AD and MCI participants (Hanzel et al., 2014). These findings have also been replicated in transgenic animal models of the AD-like amyloid pathology (Bruno et al., 2009a; Iulita et al., 2017). Further, it was suggested that there is a link between such NGF dysmetabolism and CNS inflammation in the amyloid pathology since injection of Aβ oligomers in the hippocampus of naïve rats provoked both brain inflammation and NGF dysregulation (Bruno et al., 2009a).

Interestingly, a similar NGF dysmetabolism with increased cortical proNGF levels has been reported in DS (Iulita et al., 2014, 2016; Iulita and Cuello, 2016; Caraci et al., 2017), therefore providing an explanation for the cholinergic atrophy in DS (Yates et al., 1983; Kish et al., 1989; Lubec et al., 2001). In DS as in AD, reduced levels of tPA and plasminogen, which are involved in proNGF maturation as well as heightened neuroserpin expression lead to a build-up of proNGF. In parallel, over-activation of MMP-9, the main NGF-degrading protease, leads to increased degradation of the biologically active mNGF protein (Iulita and Cuello, 2014; Iulita et al., 2014). This double hit on the NGF pathway results in decreased availability of mature NGF to sustain trophic support of BFCNs in DS as in AD. Such impairment in NGF metabolism is an early event in DS and is detectable before the clinical presentation of AD. Indeed, increased levels of proNGF, decreased tPA activity and increased MMP-9 activity were detected in conditioned media from primary cultures from fetal DS cortex (Iulita et al., 2014). In addition, levels of proNGF, as well as MMP-1, MMP-3, and MMP-9 activity were found elevated at AD asymptomatic stages in the plasma from a cohort of clinically characterized DS individuals. In this cohort, an elevation of proNGF levels at the 1-year follow-up predicted the extent of cognitive deterioration (Iulita and Cuello, 2016). The association between Aβ and NGF pathway dysfunction was further strengthened by the fact that Aβ load highly correlated with the elevation of proNGF in older DS individuals (Iulita and Cuello, 2016). The presence of an

APOEε4 allele in DS individuals, as in other people at risk of AD, may further aggravate the brain's NGF dysmetabolism. Indeed, APOEε4 mice show upregulated levels of both proMMP9 and MMP9 (Bell et al., 2012).

NGF METABOLIC PATHWAY RELATED BIOMARKERS AS INDICATORS OF AD PATHOLOGY IN DS

The diagnosis of AD in DS is challenging given the underlying DS intellectual disability and the lack of diagnostic criteria and cognitive screening tools adapted to people with DS (Lee et al., 2017). Therefore, validated biomarkers that signal the progression of Alzheimer pathology in DS are presently of great medical importance. Correlations between classical AD biomarkers and cognition are increasingly being established to define the status of this pathology in DS (Fortea et al., 2020). We propose that NGF metabolism-related biomarkers in body fluids should assist in that task.

Analysis of cortical thickness, intracranial volume, fraction anisotropy, and cerebral blood flow employing magnetic resonance imaging (MRI) could identify AD pathology in both DS and sporadic populations (Handen et al., 2020). Alternatively, positron emission tomography (PET) imaging to trace amyloid deposition with compounds such as Pittsburgh Compound B (PiB) and [18F]-florbetaben, commonly used to detect sporadic AD, have shown mixed results in identifying AD within the DS population. It was suggested that since those with DS display a lifelong amyloidoisis that is already very prominent at a young age, amyloid PET may not be of use in tracking the progress of AD (Abrahamson et al., 2019). More recently, a cross-sectional and longitudinal study in individuals with DS showed that it was possible to differentiate MCI-DS from the cognitively stable group using [18F]-AV-45 (florbetapir) PET. Additionally, although PET tracers for Tau have proved a challenge for the field (Robertson et al., 2017), a recent study using the Tau PET tracer [18F]-AV1451 in a small cohort of DS individuals showed that Tau deposition was correlated with age, amyloid deposition, decreased brain volume and reduced glucose metabolism (Rafii et al., 2017). Evaluation of Tau PET tracers using autopsy brain tissue also suggested that the regional distribution of Tau pathology in DS differs from ADAD and sporadic AD (Lemoine et al., 2020). An issue with current neuroimaging studies in DS populations is that the normative atlases being used were developed for the non-DS population, although this is currently being addressed by the creation of atlases for the DS brain (McGlinchey et al., 2020).

The pattern of biofluid biomarker changes in AD in DS have been considered to be largely similar to those in sporadic AD (Rafii et al., 2015). While those with DS have a higher baseline of Aβ peptides due to the triplication of the APP and BACE2 genes located on chromosome 21, an increase in CSF levels of Aβ42 or the Aβ42/Aβ40 ratio relative to this baseline are associated with the onset of AD in DS (Lee et al., 2017). Several studies have demonstrated that changes in plasma Aβ40 and Aβ42 in DS correlate with AD onset (Schupf et al., 2007; Jones et al., 2009; Matsuoka et al., 2009; Schupf et al., 2010; Coppus et al., 2012). As for Tau, increases in CSF total Tau (tTau) and phosphorylated Tau (pTau), have been correlated with AD onset in DS (McGlinchey et al., 2020; Pentz et al., 2021a). Likewise, plasma neurofilament light (NfL), and IL1β, have been shown in multiple studies to reliably distinguish DSAD individuals with DS asymptomatic for AD (aDS) (Petersen and O'Bryant, 2019; Startin et al., 2019; McGlinchey et al., 2020). Of the biomarkers discussed, NfL has emerged as the leading plasma biomarker. With 90% sensitivity and 92% specificity in its ability to distinguish between aDS and prodromal DSAD groups (Petersen and O'Bryant, 2019; McGlinchey et al., 2020). Additional more recently posited biofluid biomarkers include levels of TNF-a, IL-6, IL-10, and S-adenosylhomocysteine (SAH), a change in SAM/SAH ratio and CpG methylation percentage (Lee et al., 2017).

Given that degeneration of the cortical forebrain cholinergic system is a critical factor associated with cognitive decline in AD, both in the general population and in DS, as discussed above, current AD biomarker panels should be enriched by the addition of biomarkers able to monitor cholinergic dysfunction in both research and clinical contexts (Hampel et al., 2018; Cuello et al., 2019; Pentz et al., 2021a). NGF dysmetabolism's presence within DS and AD brains, and its relationship to cholinergic dysfunction, present the opportunity for the identification of novel biomarkers signifying AD pathology and subtyping for cholinergic dysfunction within DS populations. Analysis of NGF pathway proteins in matched CSF/plasma samples from DSAD and individuals with DS aDS, as well as controls, revealed that the levels of the 50 kDa isoform of proNGF and MMP9 in CSF were competent to identify symptomatic AD from the wider DS population. Both members of the NGF metabolic pathway identified symptomatic AD from the wider DS population with a sensitivity and specificity matching or outperforming that of the classical AD CSF biomarkers pTau, tTau, and the AB42/40 ratio (Pentz et al., 2021a). Importantly, longitudinal increases in 50 kDa proNGF levels in plasma over 1 year correlated to prospective cognitive decline over the subsequent 2 years (Iulita and Cuello, 2016), demonstrating a potential value of NGF-related biomarkers in identifying incipient cognitive decline in this population.

CONCLUSION

The nearly inexorable development of the AD pathology and the ensuing dementia in DS individuals is nowadays well-established and has been eloquently summarized by Lott and Head (2019). The growing awareness of this situation has triggered an increased interest and research in unraveling aspects of the AD pathology in DS, as this is the largest population of genetic AD and therefore offers clues regarding the early, preclinical stages of this pathology. A pathology which continues to defy therapeutic intervention.

As discussed in this brief review, the occurrence of NGF dysmetabolism leading to BFCNs dysfunction is now well-established. NGF metabolism-related biomarkers have proven significance in identifying AD pathology at preclinical stages and in monitoring its progression in the AD clinical continuum. This might offer distinctive possibilities of defining differential conditions of cholinergic compromise.

Alzheimer's disease is presently recognized as being the leading cause of death in DS. Therefore, novel biomarkers signaling the initial, preclinical stages of AD in DS should offer valuable tools for future early therapeutic interventions. A scenario which would spare DS individuals of the onset of clinical AD and which would also provide new therapeutic opportunities for individuals with sporadic AD.

The further investigation of the NGF metabolic compromise in AD should provide clues as to how best re-establish an adequate trophic support for the phenotypic maintenance of BFCNs; the atrophy of which importantly contributes to cognitive decline in AD pathology. If such pharmacological intervention becomes feasible it would halt the progressive atrophy of the BF cholinergic system. An effective pharmacological intervention of a deregulated NGF metabolic pathway would signify restoring mNGF homeostasis at physiological levels and at physiological sites.

AUTHOR CONTRIBUTIONS

ACC and SDC designed and outlined the structure and contents of the review. All authors contributed to the writing and revision of the manuscript and approved the submitted version.

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Postmortem Neocortical ^{3}H-PiB Binding and Levels of Unmodified and Pyroglutamate Aβ in Down Syndrome and Sporadic Alzheimer's Disease

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Individuals with Down syndrome (DS) have a genetic predisposition for amyloid-β (Aβ) overproduction and earlier onset of Aβ deposits compared to patients with sporadic late-onset Alzheimer's disease (AD). Positron emission tomography (PET) with Pittsburgh Compound-B (PiB) detects fibrillar Aβ pathology in living people with DS and AD, but its relationship with heterogeneous Aβ forms aggregated within amyloid deposits is not well understood. We performed quantitative *in vitro* ^{3}H-PiB binding assays and enzyme-linked immunosorbent assays of fibrillar (insoluble) unmodified Aβ40 and Aβ42 forms and *N*-terminus truncated and pyroglutamate-modified AβNpE3-40 and AβNpE3-42 forms in postmortem frontal cortex and precuneus samples from 18 DS cases aged 43–63 years and 17 late-onset AD cases aged 62–99 years. Both diagnostic groups had frequent neocortical neuritic plaques, while the DS group had more severe vascular amyloid pathology (cerebral amyloid angiopathy, CAA). Compared to the AD group, the DS group had higher levels of Aβ40 and AβNpE3-40, while the two groups did not differ by Aβ42 and AβNpE3-42 levels. This resulted in lower ratios of Aβ42/Aβ40 and AβNpE3-42/AβNpE3-40 in the DS group compared to the AD group. Correlations of Aβ42/Aβ40 and AβNpE3-42/AβNpE3-40 ratios with CAA severity were strong in DS cases and weak in AD cases. Pyroglutamate-modified Aβ levels were lower than unmodified Aβ levels in both diagnostic groups, but within group proportions of both pyroglutamate-modified Aβ forms relative to both unmodified Aβ forms were lower in the DS group but not in the AD group. The two diagnostic groups did not differ by ^{3}H-PiB binding levels. These results demonstrate that compared to late-onset AD cases, adult DS individuals with similar severity of neocortical neuritic plaques and greater CAA pathology have a preponderance of both pyroglutamate-modified AβNpE3-40 and unmodified Aβ40 forms. Despite the distinct molecular profile of Aβ forms and greater vascular amyloidosis in DS cases, cortical ^{3}H-PiB binding does not distinguish between

diagnostic groups that are at an advanced level of amyloid plaque pathology. This underscores the need for the development of CAA-selective PET radiopharmaceuticals to detect and track the progression of cerebral vascular amyloid deposits in relation to Aβ plaques in individuals with DS.

Keywords: Alzheimer's disease, amyloid, cerebral amyloid angiopathy, default mode network, Down syndrome, Pittsburgh Compound-B, pyroglutamate

INTRODUCTION

Individuals with Down syndrome (DS) have an overabundance of amyloid-β (Aβ) peptide production due to trisomy of chromosome 21, which harbors the Aβ-precursor protein (APP) gene (Oyama et al., 1994), and they typically develop Alzheimer's disease (AD) pathology by the fifth decade of life (Davidson et al., 2018). The primary histopathological features of AD that are present in the DS brain include amyloid plaque deposits of fibrillar Aβ peptides and neurofibrillary tangles of over-phosphorylated tau protein (Wisniewski et al., 1985; Mann, 1988; Dickson, 2005; Head et al., 2016; Davidson et al., 2018; Perez et al., 2019). As in AD, fibrillar Aβ also accumulates in the brain vasculature (cerebral amyloid angiopathy, CAA) in the DS brain, at levels exceeding those seen in normal aging (Vinters, 1987; Carmona-Iragui et al., 2017; Head et al., 2017; Davidson et al., 2018).

Recent improvements in medical care have contributed to the increased longevity of individuals with DS, and advancements in diagnostic biomarkers have facilitated studies of key questions regarding the interconnected clinical and neuropathological features that develop with age in DS (Neale et al., 2018; Handen et al., 2020; Head and Ances, 2020; Petersen et al., 2020, 2021; Rafii et al., 2020; Hendrix et al., 2021). Positron emission tomography (PET) studies using Pittsburgh Compound-B (^{11}C-PiB) and related amyloid-binding radiopharmaceuticals provide insight into regional distributions and temporal changes in Aβ pathology in living people with AD (Klunk et al., 2004; Rowe et al., 2007; Cohen et al., 2012; Mathis et al., 2017; Villemagne et al., 2021) and this technology is being applied increasingly to studies of individuals with DS (Landt et al., 2011; Handen et al., 2012; Hartley et al., 2014, 2017, 2020; Annus et al., 2016, 2017; Lao et al., 2016, 2017, 2018; Cole et al., 2017; Cohen et al., 2018; Neale et al., 2018; Mak et al., 2019a,b; Mihaila et al., 2019; Tudorascu et al., 2019, 2020; Wilson et al., 2019; Cody et al., 2020; Zammit et al., 2020, 2021). PET imaging of brain Aβ pathology and brain metabolism as well as functional connectivity (fMRI) studies have shown that certain brain regions are more vulnerable than others to pathological changes in AD. Cortical association areas contributing to the core regions of the default mode network (DMN), including the frontal cortex, the precuneus, and the posterior cingulate cortex, show functional impairment and amyloid deposition in early AD stages (Buckner et al., 2005; Jones et al., 2011; Palmqvist et al., 2017), and these brain regions are also affected in adults with DS (Tudorascu et al., 2019; Wilson et al., 2019) suggesting that amyloid pathology affects similar cortical circuits in DS and AD. A recent longitudinal ^{11}C-PiB PET study reported slower progression of the frontal cortex and precuneus amyloid pathology in nondemented young adults with DS (mean age 37 years) when compared to nondemented elderly (mean age 73 years; Tudorascu et al., 2019). Thus, there is a need for determining if amyloid PET ligand retention is influenced by regional differences in structural and biochemical characteristics of Aβ pathology in DS compared to aging and AD.

Autopsy studies demonstrated that cyano-PiB, a highly fluorescent derivative of PiB which detects Aβ plaques in histological sections from AD brains (Ikonomovic et al., 2008, 2020), also labels Aβ plaques in postmortem DS brain tissue (LeVine et al., 2017; Abrahamson et al., 2019; Perez et al., 2019). In addition, analyses of *in vitro* binding of ^{3}H-PiB to postmortem frontal cortex homogenates showed that in DS individuals higher binding levels were associated with more advanced age (LeVine et al., 2017) and that DS individuals between the ages of 43–63 years had significantly higher binding levels compared to cognitively normal elderly between the ages of 78–92 years and cases with mild-moderate AD between the ages of 77–101 years (Abrahamson et al., 2019). However, the contribution of molecularly heterogeneous Aβ forms (Saido et al., 1996; Roher et al., 2017), and their conformational changes when fibrillized (Schlenzig et al., 2009; Chen et al., 2017; Creekmore et al., 2021), on PiB binding is not well understood. Aβ peptides with the C-terminus ending at amino acid 42 predominate in Aβ plaques (Dickson, 1997), and are believed to be the initially deposited and a principal Aβ form in Aβ plaques in both AD and DS (Jarrett et al., 1993; Iwatsubo et al., 1994, 1995; Saido et al., 1995; Mann and Iwatsubo, 1996; Michno et al., 2019; Golde et al., 2000). In contrast, Aβ peptides with the C-terminus ending at the amino acid 40 are more soluble and are reported to be more prevalent in vascular Aβ deposits (CAA) than in parenchymal Aβ plaques (Jarrett et al., 1993; Miller et al., 1993; Gravina et al., 1995; Iwatsubo et al., 1995; Akiyama et al., 1997; Harigaya et al., 2000; Guntert et al., 2006; Mann et al., 2018; Gkanatsiou et al., 2019). In addition, modified Aβ forms with *N*-terminus truncations are a significant proportion of total plaque-bound Aβ in AD and aged DS brains (Masters et al., 1985). *N*-terminus truncated Aβ can be modified further by the enzyme glutaminyl cyclase into forms with pyroglutamate at the 3rd amino acid (AβNpE3) or the 11th amino acid (AβNpE11; Cynis et al., 2008; Schilling et al., 2008; Morawski et al., 2104). Pyroglutamate-modified Aβ forms are believed to play a role in seeding or maturation of Aβ plaques; they are more resistant to proteolytic cleavage by peptidases, which may impede their clearance, and *in vitro* they accelerate fibril formation of unmodified forms (Saido et al., 1995; He and Barrow, 1999; Schilling et al., 2006; Gunn et al.,

2010; Jawhar et al., 2011; Sullivan et al., 2011; Dammers et al., 2017; Michno et al., 2019). Both AβNpE3 and AβNpE11 forms contribute to Aβ plaques, however, AβNpE11 is restricted mainly to the innermost amyloid core (Sullivan et al., 2011) where it may be less accessible to peptidases as well as PET radioligands. The clinical significance of pyroglutamate Aβ is not known. Recent studies reported that high levels of insoluble Aβ42 forms, including AβNpE3-42, correlated with cognitive impairment across clinical stages of AD (Pivtoraiko et al., 2015; Abrahamson et al., 2016). Passive immunization with a pyroglutamate-3 Aβ IgG1 monoclonal antibody reduced amyloid plaque burden and improved behavior in APPswe/PS1ΔE9 mice (Frost et al., 2015). In a Phase 2 clinical trial of early AD, treatment with donanemab, a humanized IgG1 monoclonal antibody developed from the mouse monoclonal antibody mE8-IgG2a (Demattos et al., 2012) and specific for AβNpE3-42, reduced Aβ plaque burden and slowed cognitive decline (Mintun et al., 2021). Thus, AβNpE3-42 may be an important substrate for amyloid PET ligand retention, a biomarker for brain amyloidosis, and a therapeutic target.

In adults with DS, pyroglutamate-modified Aβ immunoreactivity was demonstrated in cortical Aβ plaques at the ages 30–40 years (but not younger), with greater abundance at ages 50–70 years (Lemere et al., 1996; Frost et al., 2013). Studies have also compared N-terminally truncated, pyroglutamate-modified Aβ forms to other forms of Aβ in DS brains using biochemical methods (Saido et al., 1995; Russo et al., 1997; Hosoda et al., 1998; Gkanatsiou et al., 2021) but none in relation to binding of PiB or related amyloid PET radioligands. In the current study, we quantified fibrillar forms of unmodified Aβ (Aβ42 and Aβ40) as well as pyroglutamate-modified Aβ (AβNpE3-42 and AβNpE3-40) and *in vitro* binding levels of ^{3}H-PiB (as a proxy for PiB PET imaging) in postmortem homogenates of the frontal cortex and precuneus gray matter from a group of older adults with DS (age range: 43–63 years) compared to a group of sporadic AD cases (age range: 62–99 years) with a comparable degree of AD neuropathologic change.

MATERIALS AND METHODS

Subjects

Frozen postmortem brain tissue specimens from the frontal cortex and the precuneus were obtained from 18 DS cases, provided by the University of California, Irvine Alzheimer's Disease Research Center (UCI-ADRC) and Institute for Memory Impairments and Neurological Disorders, and from 17 sporadic AD cases in the University of Pittsburgh Alzheimer's Disease Research Center (ADRC) brain bank. Clinical diagnosis of AD dementia utilized standard criteria (McKhann et al., 1984). Brain autopsy consent was obtained under a protocol approved by the Institutional Review Boards and the use of autopsy tissue for research was approved by the Committee for Oversight of Research and Clinical Training Involving Decedents (CORID) at the University of Pittsburgh and the University of California, Irvine. DS and AD brains were assessed for neocortical neuritic plaques and neurofibrillary pathology according to the National Institute on Aging-Alzheimer's Association guidelines (Montine et al., 2012), using the Consortium to Establish a Registry for Alzheimer's disease (CERAD) neuritic plaque scoring protocol (Mirra et al., 1991) and Braak staging for neurofibrillary pathology (Braak and Braak, 1991; Braak et al., 2006). The severity of CAA was evaluated separately in the frontal cortex and in the precuneus in both diagnostic groups, using Aβ immunohistochemistry with mouse monoclonal IgG clone NAB228 (37-4200, Thermo-Fisher, Waltham, MA) on 4% paraformaldehyde fixed tissue sections, on a four-point rating scale (0, none; 1, mild; 2, moderate; 3, severe) by two independent evaluators (EA and MI) adapted from published studies (Olichney et al., 1995; Arvanitakis et al., 2011). Demographic and neuropathological information of cases are detailed in **Table 1**. Frozen frontal cortex was not available for one AD case (AD-9). Frozen precuneus samples were not available for two DS cases (DS-10 and DS-11) and two AD cases (AD-8 and AD-10).

Tissue Preparation

Frozen gray matter samples were homogenized in 0.01 M sodium phosphate-buffered saline (PBS, pH 7.4) to a concentration of 300 mg wet brain tissue/mL. This homogenate is referred to as a "whole (unfractionated) tissue homogenate" and was used for the ^{3}H-PiB binding assay. A protease inhibitor cocktail (AEBSF: 104 mM, aprotinin at 80 μM, bestatin at 4 mM, E-64 at 1.4 mM, leupeptin at 2 mM and pepstatin A at 1.5 mM; P8340, Sigma, St. Louis, MS; used at a 1:100 dilution) was added, and samples were then centrifuged at 100,000× *g* for 1 h at 4°C. The pellet was sonicated in 70% formic acid to solubilize Aβ fibrils. Samples were then centrifuged at 113,000× *g* for 1 h at 4°C. The supernatant containing the extracted, solubilized fibrillar Aβ fraction (hereafter referred to as "insoluble Aβ" and assayed by the ELISA) was then removed and neutralized to pH 7.4, divided into aliquots, and frozen at −80°C until testing was performed.

Quantification of Aβ Peptide Levels

Solid-phase sandwich ELISA kits were used to measure AβNpE3-42 and AβNpE3-40 peptide levels (27716 and 27418, Immuno-Biological Laboratories, Minneapolis, MN). The AβNpE3-42 assay utilized a plate precoated with a capture antibody against the Aβ carboxy-terminal amino acid 42 (anti-human Aβ 38–42 rabbit polyclonal IgG). The AβNpE3-40 assay utilized a plate precoated with a capture antibody against the Aβ carboxy-terminal amino acid 40 (anti-human Aβ 35–40 mouse monoclonal IgG). A detection antibody for human AβNpE3 [anti-human AβN3pE (clone 8E1) mouse monoclonal IgG] was used in both assay kits. Solid phase sandwich ELISA kits were used to measure unmodified Aβ42 and Aβ40 peptide levels (KHB3441 and KHB3481, Thermo) on a plate precoated with a capture antibody directed against the unmodified amino terminus of Aβ, and detection antibodies specific for Aβ42 or Aβ40, respectively. Procedures were followed as outlined in the manufacturer's instructions. Optical density values were read at 450 nm with a plate reader (SpectraMax i3x, Molecular Devices, San Jose, CA, USA) using SoftMax Pro software, Version 6.5.1 (Molecular Devices). Results were determined from standard curves that used synthetic human AβNpE3-40, AβNpE3-42,

TABLE 1 | Demographic and neuropathological characteristics of Down syndrome and Alzheimer's disease cases.

Case code	Age (years)	Sex (M/F)	Neocortical neuritic plaques	Braak stage	CAA severity (frontal cortex)	CAA severity (precuneus cortex)
			Down syndrome			
DS-1	49	M	Frequent	VI	None	None
DS-2	43	M	Frequent	VI	None	None
DS-3	57	F	Frequent	VI	None	Severe
DS-4	62	F	Frequent	VI	Mild	None
DS-5	45	F	Frequent	VI	Mild	Mild
DS-6	50	M	Frequent	VI	Mild	Mild
DS-7	57	F	Frequent	VI	Mild	Moderate
DS-8	55	F	Frequent	VI	Mild	Moderate
DS-9	52	F	Frequent	VI	Mild	Severe
DS-10	56	M	Frequent	VI	Moderate	Mild
DS-11	56	F	Frequent	VI	Moderate	Mild
DS-12	49	M	Frequent	VI	Moderate	Mild
DS-13	55	M	Frequent	VI	Moderate	Mild
DS-14	46	M	Frequent	VI	Moderate	Moderate
DS-15	50	F	Frequent	VI	Severe	Moderate
DS-16	63	F	Frequent	VI	Severe	Moderate
DS-17	58	M	Frequent	VI	Severe	Severe
DS-18	54	M	Frequent	VI	Severe	Severe
			Alzheimer's disease			
AD-1	85	M	Frequent	III/IV	None	None
AD-2	89	F	Frequent	VI	None	None
AD-3	91	M	Frequent	V	None	None
AD-4	77	M	Frequent	VI	None	Mild
AD-5	74	M	Frequent	VI	Mild	None
AD-6	67	F	Frequent	VI	Mild	None
AD-7	99	M	Frequent	V	Mild	None
AD-8	82	M	Frequent	VI	Mild	None
AD-9	91	F	Frequent	VI	Mild	None
AD-10	85	M	Frequent	VI	Mild	Mild
AD-11	62	M	Frequent	VI	Mild	Mid
AD-12	79	M	Frequent	VI	Mild	Mild
AD-13	84	M	Frequent	VI	Mild	Moderate
AD-14	77	M	Frequent	VI	Moderate	None
AD-15	88	M	Frequent	V	Moderate	Mild
AD-16	72	M	Frequent	VI	Severe	Moderate
AD-17	76	M	Frequent	V	Severe	Severe

Aβ40, and Aβ42 and are expressed as picomoles per gram of wet tissue weight. Samples were run in duplicates, including both diagnostic groups and both brain regions in each experiment. Each sample was analyzed at least twice and the mean of the two assays was used to determine final values for each sample/analyte.

^{3}H-PiB Binding Assay

Unfractionated whole brain tissue homogenates (described above) were diluted from 300 mg/ml to a concentration 10 mg/ml in PBS prior to the binding assay as previously described (Ikonomovic et al., 2008) with the exception of the fold-higher initial homogenate prepared in the current study. For determination of ^{3}H-PiB binding, 1 nM ^{3}H-PiB (American Radiolabeled Chemicals, St. Louis, MO, USA; specific activity 72.4 Ci/mmol) was incubated with 100 μg tissue in 1 ml PBS as described previously (Ikonomovic et al., 2008). Unlabeled PiB was dissolved in DMSO at 400 mM (to yield 51% DMSO) and this stock solution was diluted with PBS to achieve the desired concentration for the binding assay. Non-specific binding was defined as the number of counts remaining in the presence of 1 mM unlabeled PiB. The binding mixtures were filtered through a Whatman GF/B glass filter using a Brandel M24R cell harvester (Brandel, Gaithersburg, MD) and rapidly washed five times with 3 ml PBS. The filters were counted in Cytoscint-ES after thorough vortex mixing and resting overnight. Results were corrected for non-specific, non-displaceable binding in the presence of 1 mM PiB and expressed as picomoles ^{3}H-PiB bound per gram of wet brain tissue weight in the homogenate.

Statistical Analysis

Statistical analysis and graphs were performed using GraphPad PRISM Version 8 software (GraphPad, San Diego, CA, USA). The Kruskal-Wallis one-way analysis of variance was used to compare groups and pairwise comparisons were performed using Dunn's multiple comparisons post test. The Spearman rank order correlation test was used to assess associations between two variables. Demographic and diagnostic neuropathological characteristics in the DS group were compared to the AD group

using Student's *t*-test and chi-square tests where appropriate. Significance was set at $P < 0.05$.

RESULTS

Case Demographics and Neuropathological Characteristics

Individual case demographics and neuropathological characteristics are listed in **Table 1**. The DS group on average was younger than the AD group (DS: 53 ± 6 years; AD: 84 ± 10 years, $P < 0.001$; **Table 1**). Females were more represented in the DS group (nine females and nine males; 50%; $p < 0.01$) compared to the AD group (three females and 14 males; 18%).

The severity of AD neuropathological changes was similar between the two groups when compared by CERAD scores or by Braak staging, with all cases in the study having frequent neocortical neuritic plaques as well as neocortical stages of neurofibrillary pathology (Braak stages V or VI) with the exception of one AD case (AD-1) that was determined to be Braak stage III/IV (**Table 1**). The severity rating of CAA pathology was higher in the precuneus ($p = 0.0156$) and trended higher in the frontal cortex ($p = 0.1990$) in the DS group compared to the AD group (**Table 1**).

Diagnostic Group and Brain Region Comparisons: ^{3}H-PiB Binding, Unmodified Aβ42 and Aβ40, Pyroglutamate AβNpE3–42 and AβNpE3–40; Ratios of Aβ42/Aβ40 and AβNpE3–42/AβNpE3–40

in vitro ^{3}H-PiB binding levels in the frontal cortex and in the precuneus in the DS group did not differ from ^{3}H-PiB binding levels in the same regions, respectively, in the AD group (**Figure 1**, **Table 2**).

In both the frontal cortex and the precuneus, levels of unmodified Aβ42 and pyroglutamate AβNpE3-42 were not statistically different between DS and AD groups (**Figures 2A–C**, **Table 2**). Unmodified Aβ40 and pyroglutamate AβNpE3-40 levels in the frontal cortex and in the precuneus were significantly higher in the DS group compared to the AD group (**Figures 2B,D**, **Table 2**). The ratios of unmodified Aβ42/Aβ40 levels and pyroglutamate AβNpE3-42/AβNpE3-40 levels in the frontal cortex and in the precuneus were significantly lower in the DS group compared to the AD group (**Figures 3A,B**, **Table 2**).

Comparisons of Unmodified and Pyroglutamate-Modified Aβ Forms Within Each Brain Region in Each Diagnostic Group

In both the frontal cortex and the precuneus from the DS group, unmodified Aβ42 and Aβ40 were at similar levels and both were higher than AβNpE3-42 and AβNpE3-40 levels, which were also at similar levels in this group (**Table 2**). In the frontal cortex and in the precuneus from the AD group, unmodified Aβ42 levels were higher than Aβ40, AβNpE3-40, and AβNpE3-42 levels, and both Aβ40 and AβNpE3-42 levels were higher than AβNpE3-40 levels (**Table 2**).

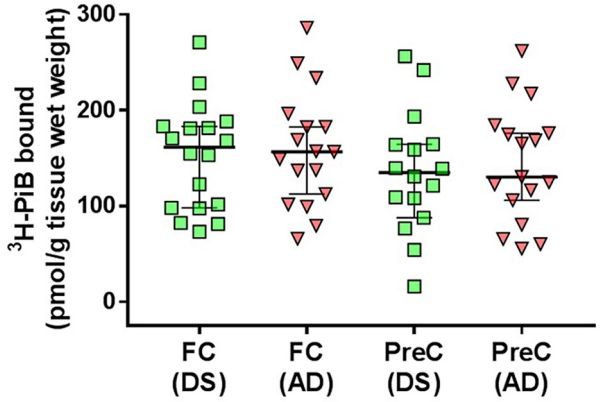

FIGURE 1 | ^{3}H-PiB binding levels in tissue homogenates of the frontal cortex (FC) and precuneus (PreC) obtained postmortem from individuals with Down syndrome (DS) or Alzheimer's disease (AD). Individual data points and group medians with 95% confidence intervals are illustrated in the graphs. Data were assessed using the Kruskal-Wallis one-way analysis of variance with Dunn's multiple comparisons post test.

Ratios of Aβ42/AβNpE3–42 and Aβ40/AβNpE3–40 in the DS Group Compared to the AD Group

In the DS group, the ratio of Aβ42/AβNpE3-42 in the frontal cortex was higher, while in the precuneus the ratio trended higher, compared to the AD group (**Figure 3C**, **Table 2**). The ratio of Aβ40/AβNpE3-40 levels in the precuneus was higher in the DS group compared to the AD group, while in the frontal cortex the ratio trended higher in the DS group (**Figure 3D**, **Table 2**).

Associations of Unmodified Aβ Levels With Pyroglutamate-Modified Aβ Levels, Both Aβ Forms With ^{3}H-PiB Binding Levels, and Both Aβ Forms and ^{3}H-PiB Binding Levels With CAA Severity in Down Syndrome and Alzheimer's Disease Groups

For correlation analyses within each diagnostic group, data from the frontal cortex and the precuneus were combined. We observed significant associations between levels of Aβ42 and AβNpE3-42 and between levels of Aβ40 and AβNpE3-40 in each diagnostic group (**Table 3**).

There was a significant association of unmodified Aβ42 levels with ^{3}H-PiB levels in the DS group and a similar trend was present in the AD group (**Table 3**). No associations were observed between unmodified Aβ40 levels and ^{3}H-PiB levels in either diagnostic group. We observed significant associations of AβNpE3-42 levels with ^{3}H-PiB levels in both diagnostic groups and a significant association of AβNpE3-40 levels with ^{3}H-PiB levels in the DS group but not in the AD group (**Table 3**).

TABLE 2 | Comparisons of ^{3}H-PiB, unmodified Aβ, pryoglutamate modified Aβ, and ratios of Aβ forms across groups/regions and with each other.

Variable	Down syndrome (frontal cortex)	Alzheimer's disease (frontal cortex)	Down syndrome (precuneus)	Alzheimer's disease (precuneus)	Kruskal–Wallis statistic and *P* value	Pairwise comparisons ($P < 0.05$)
^{3}H-PiB binding	152.2 ± 55.60 (161.4)	158.5 ± 59.94 (156.6)	135.1 ± 63.1 (134.9)	143.4 ± 60.86 (130.4)	1.35, $P = 0.7128$	n.s.
Aβ42	1,428 ± 373.2 (1,454)	1,274 ± 411.5 (1,252)	1,461 ± 534.3 (1,454)	1,119 ± 549.8 (919.5)	6.39, $P = 0.0943$	n.s.
Aβ40	2,868 ± 2,940 (1,337)	655.5 ± 893.0 (104.6)	5,149 ± 6,091 (2,261)	227.5 ± 404.5 (48.23)	28.46, $P < 0.0001$	FC-DS, PreC-DS > FC-AD, PreC-AD
AβNpE3-42	94.37 ± 14.97 (91.74)	90.5 ± 21.22 (90.49)	87.28 ± 16.22 (86.46)	81.25 ± 31.22 (83.25)	4.33, $P = 0.2288$	n.s.
AβNpE3-40	173 ± 208.3 (51.63)	33.26 ± 49.25 (8.38)	122.1 ± 147 (48.71)	33.1 ± 55.48 (5.88)	15.08, $P = 0.0017$	FC-DS, PreC-DS > FC-AD, PreC-AD
Kruskal–Wallis statistic and *P* value	47.65, $P < 0.0001$	32.63, $P < 0.0001$	41.83, $P < 0.0001$	36.40, $P < 0.0001$		
Pairwise comparisons ($P < 0.05$)	Aβ42, Aβ40 > AβNpE3-42, AβNpE3-40	Aβ42 > Aβ40, AβNpE3-42, AβNpE3-40; Aβ40, AβNpE3-42 > AβNpE3-40	Aβ42, Aβ40 > AβNpE3-42, AβNpE3-40	Aβ42 > Aβ40, AβNpE3-40, AβNpE3-42; Aβ40, AβNpE3-42 > AβNpE3-40		
Aβ42/Aβ40	1.69 ± 1.82 (1.04)	13.29 ± 17.29 (4.0)	2.05 ± 3.05 (0.54)	43.44 ± 51.0 (13.95)	20.73, $P = 0.0001$	FC-DS, PreC-DS > FC-AD, PreC-AD
AβNpE3-42/AβNpE3-40	2.69 ± 3.05 (1.68)	18.98 ± 19.32 (12.61)	4.554 ± 6.939 (1.095)	29.37 ± 29.07 (10.12)	13.36, $P = 0.0035$	FC-DS, PreC-DS > FC-AD, PreC-AD
Aβ42/AβNpE3-42	16.01 ± 2.12 (15.86)	13.92 ± 4.40 (13.51)	15.83 ± 4.22 (15.11)	14.50 ± 8.37 (11.30)	10.0, $P = 0.0185$	FC-DS > FC-AD, PreC-AD
Aβ40/AβNpE3-40	25.73 ± 30.43 (18.72)	15.69 ± 10.38 (13.02)	71.43 ± 153.7 (33.16)	10.19 ± 11.96 (8.48)	28.46, $P < 0.0001$	PreC-DS > FC-AD FC-DS > PreC-AD; PreC-DS > FC-AD, PreC-AD

Horizontal comparisons: AβNpE3-40, AβNpE3-42, and the AβNpE3-42/AβNpE3-40 ratio, Aβ40, Aβ42, and the Aβ42/Aβ40 ratio, and ^{3}H-PiB binding levels in Down syndrome compared to Alzheimer's disease in the frontal cortex and in the precuneus. Vertical comparisons: AβNpE3-40, AβNpE3-42, Aβ40, and Aβ42 levels separately in the frontal cortex (FC) and precuneus cortex (PreC) in Down syndrome and in Alzheimer's disease. Units are pmol/g tissue wet weight and arithmetic means ± standard deviations and medians are shown. Comparisons were made using the Kruskal–Wallis one-way analysis of variance with Dunn's post test for multiple comparisons.

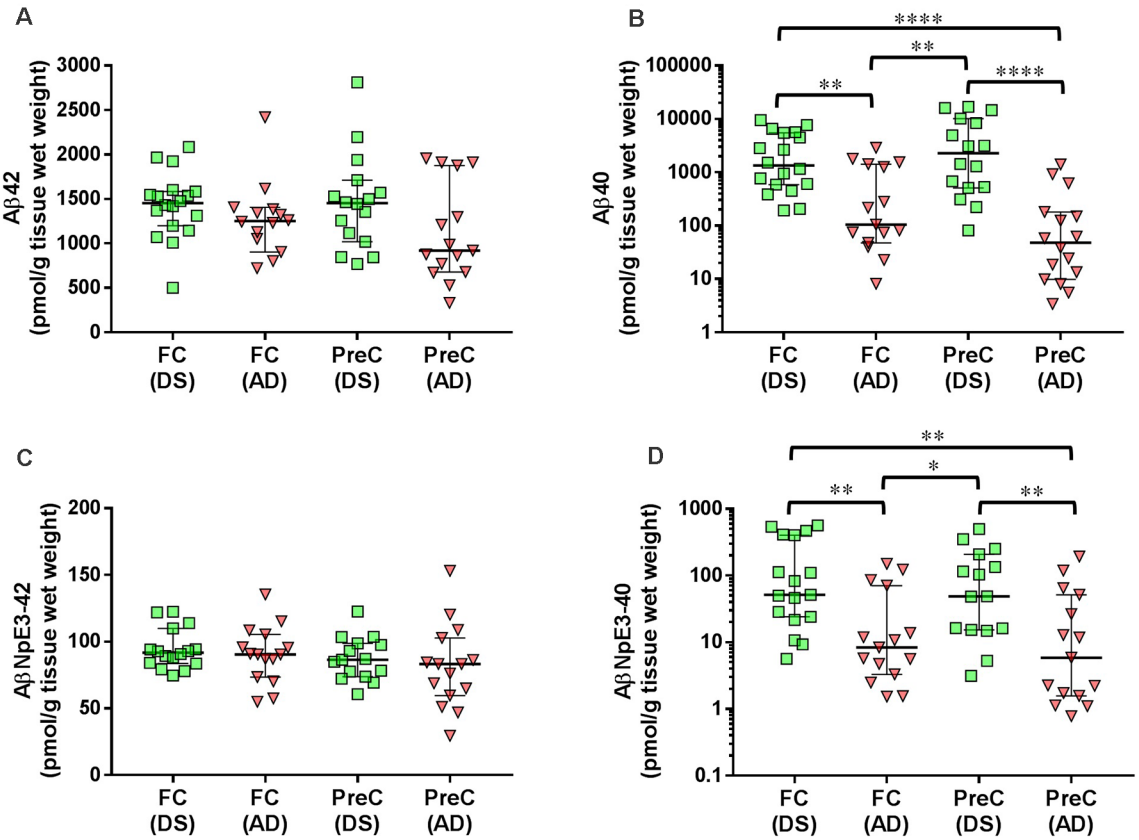

FIGURE 2 | The concentration of Aβ42 **(A)**, Aβ40 **(B)**, AβNpE3-42 **(C)**, and AβNpE3-40 **(D)** in tissue homogenates of the frontal cortex (FC) and precuneus (PreC) obtained postmortem from individuals with Down syndrome (DS) or Alzheimer's disease (AD). Individual data points and group medians with 95% confidence intervals are illustrated in the graphs. The y-axis in panels B and D have been log transformed to better illustrate the spread of individual data points. Data were assessed using the Kruskal-Wallis one-way analysis of variance with Dunn's multiple comparisons post test. Significant differences between the two groups that were identified by the post test are indicated by brackets and asterisks. *$P < 0.05$; **$P < 0.01$; ****$P < 0.0001$.

No associations were observed between the ratio of unmodified Aβ forms and ^{3}H-PiB levels in either diagnostic group. There was a significant association of the ratio AβNpE3-42/AβNpE3-40 with ^{3}H-PiB levels in the DS group but not in the AD group (**Table 3**).

In both diagnostic groups, there were significant associations of Aβ40 and AβNpE3-40 levels with CAA severity and of ratios Aβ42/Aβ40 and AβNpE3-42/AβNpE3-40 with CAA severity (**Table 3**). Greater CAA severity correlated with higher ^{3}H-PiB binding levels in the DS group, but not in the AD group (**Table 3**).

DISCUSSION

The extent of interaction between PiB, or related amyloid-binding radiopharmaceuticals for PET imaging, with different unmodified Aβ forms or post-translationally truncated and pyroglutamate-modified Aβ forms in pathological amyloid deposits in cortical regions from DS and AD brains is not well understood. Better characterization of these interactions could facilitate the interpretation of amyloid PET imaging studies and identify targets for therapy. DS patients develop amyloid pathology and are likely to show positive amyloid PET scans, by age 40. Since the pyroglutamate modification is believed to drive Aβ fibrillization and deposition in amyloid plaques (Jawhar et al., 2011), in the present study we undertook a quantitative ELISA analysis of insoluble (fibrillar) pools of unmodified Aβ42 and Aβ40 forms as well as *N*-terminus truncated and pyroglutamate-modified AβNpE3-42 and AβNpE3-40 forms in the frontal cortex and the precuneus from adult DS cases compared to a group of sporadic AD cases with similar levels of neocortical neuritic plaques and neurofibrillary tangle pathology. Additionally, we assayed ^{3}H-PiB binding levels in the same homogenates used for the ELISA studies. We found that in both the frontal cortex and the precuneus regions, Aβ42, AβNpE3-42, and ^{3}H-PiB binding levels did not differ significantly between the two diagnostic groups. In contrast, DS cases had significantly higher Aβ40 and AβNpE3-40 levels, and lower Aβ42/Aβ40 and AβNpE3-42/AβNpE3-40 ratios in these cortical regions, compared to AD cases.

Genetic predisposition for premature pathological aging with early-onset of Aβ plaque accumulation in DS relative to sporadic AD (Teller et al., 1996; Mori et al., 2002; Zigman et al., 2002, 2008) could result in a greater abundance of fibrillar Aβ deposits in brains of adults with DS when compared to individuals in the

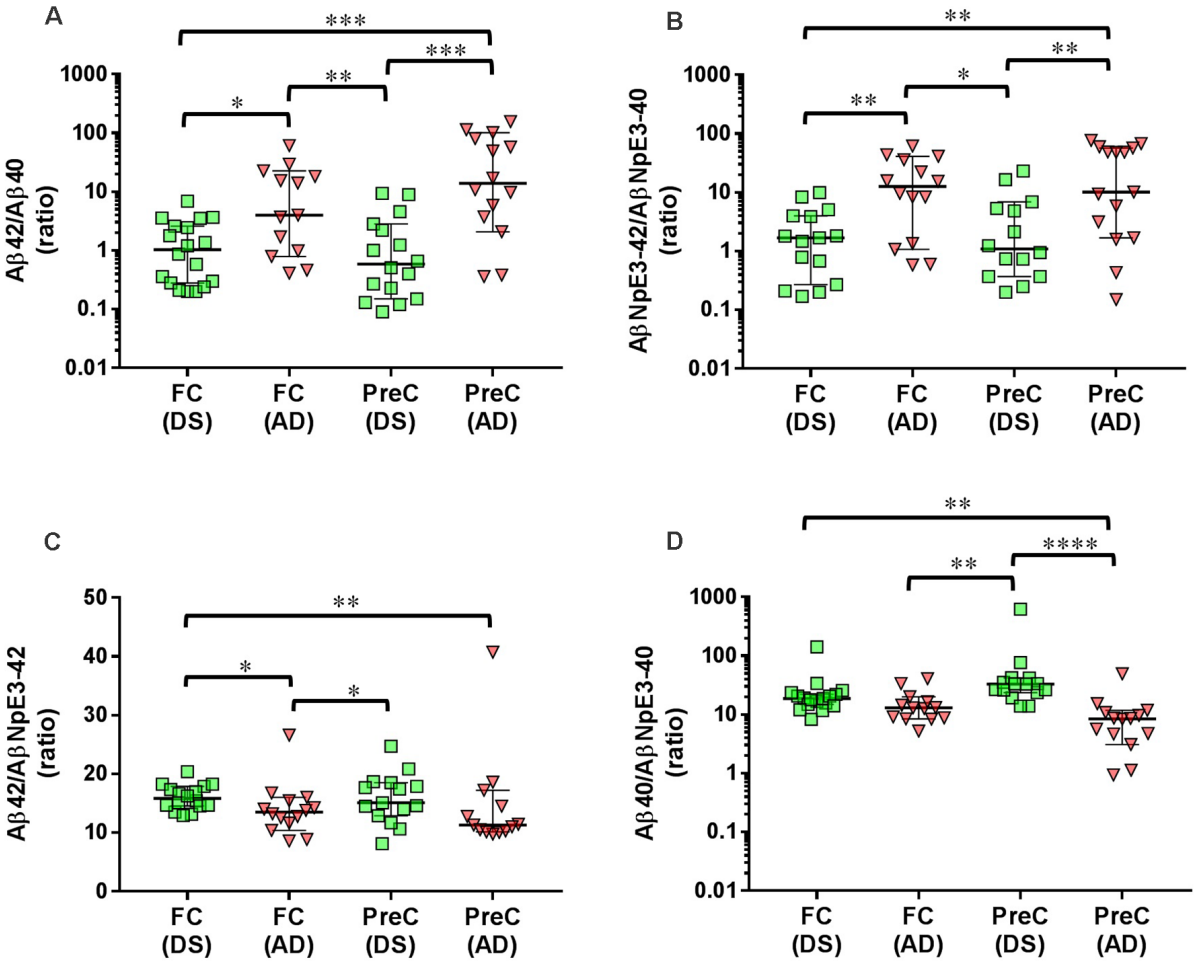

FIGURE 3 | Ratios of unmodified Aβ42/Aβ40 **(A)** and pyroglutamate AβNpE3-42/NpE3–40 **(B)**, and ratios of unmodified and pyroglutamate forms of Aβ ending at amino acid 42 **(C)** or at amino acid 40 **(D)** in tissue homogenates of the frontal cortex (FC) and precuneus (PreC) obtained postmortem from individuals with Down syndrome (DS) and Alzheimer's disease (AD). Individual data points and group medians with 95% confidence intervals are illustrated in the graphs. The y-axis in panels A, B, and D have been log transformed to better illustrate the spread of individual data points. The data were assessed using the Kruskal-Wallis one-way analysis of variance with Dunn's multiple comparisons post test. Significant differences between the two groups that were identified by the post test are indicated by brackets and asterisks. *$P < 0.05$; **$P < 0.01$; ***$P < 0.01$; ****$P < 0.0001$.

early stages of AD. In agreement with this, we previously reported higher levels of insoluble unmodified Aβ42, and a greater burden of mature amyloid plaques, in the frontal cortex from adult individuals with DS (age range: 43–63 years) compared to cases with mild-moderate AD from the Rush Religious Order Study (age range: 77–101 years; Abrahamson et al., 2019). In contrast, our current study demonstrated that in cases from the same DS cohort, neocortical levels of insoluble unmodified Aβ42 were not different from a group of late-stage AD cases in our ADRC autopsy cohort (age range: 62–99 years). Cortical levels of insoluble pyroglutamate AβNpE3-42 were also similar between the DS group and the late stage AD group in the current study. The propensity for the Aβ forms ending at amino acid 42 to aggregate into amyloid fibrils and deposit early in the process of amyloid plaque formation could explain these observations. Specifically, our DS subjects were above the age of 40 years and exhibited a high level of amyloid plaque pathology by CERAD scores for neocortical neuritic plaques that were similar to late-stage AD (Wisniewski et al., 1985; Mann and Esiri, 1989), thus it is possible that levels of fibrillar Aβx-42 forms reach a plateau early in the pathological progression of DS and AD. In contrast, Aβx-40 forms clearly distinguished DS and AD groups in our current study; in both cortical regions examined, we observed significantly higher levels of Aβ40 and AβNpE3-40 in the DS group compared to the late-stage AD group and, as a result, DS cases had lower ratios of Aβ42/40 and AβNpE3-42/AβNpE3-40 when compared to late-stage AD cases. Higher levels of Aβ40 and AβNpE3-40 in the frontal cortex of DS cases compared to AD cases were also reported in a study of five DS individuals (age range: 53–67) and 14 AD cases (age 66–86; Hosoda et al., 1998). Another major finding of our current study is that compared to AD, the DS group had lower proportions of pyroglutamate relative to unmodified Aβ forms. This could be explained by a shorter residence time

TABLE 3 | Associations of unmodified Aβ levels with pyroglutamate-modified Aβ levels, both Aβ forms with ^{3}H-PiB binding levels, and both Aβ forms with CAA severity in Down syndrome and Alzheimer's disease groups.

Comparison/Group	Down syndrome	Alzheimer's disease
Unmodified Aβ to pyroglutamate-modified Aβ (same *C*-terminus)	***Spearman r (P* value)**	***Spearman r (P* value)**
Aβ42 and AβNpE3-42	*0.5476 (0.0014)*	*0.6408 (<0.0001)*
Aβ40 and AβNpE3-40	*0.8292 (<0.0001)*	*0.8626 (<0.0001)*
Aβ forms to ^{3}H-PiB	***Spearman r (P* value)**	***Spearman r (P* value)**
Aβ42 and ^{3}H-PiB	*0.4197 (0.0135)*	*0.2852 (0.1337)*
Aβ40 and ^{3}H-PiB	0.2825 (0.1055)	−0.0097 (0.9588)
AβNpE3-42 and ^{3}H-PiB	*0.3661 (0.0428)*	*0.4459 (0.0135)*
AβNpE3-40 and ^{3}H-PiB	*0.4377 (0.0122)*	−0.0269 (0.8877)
Aβ42/Aβ40 ratio and ^{3}H-PiB	−0.2610 (0.1360)	0.2717 (0.1704)
AβNpE3-42/AβNpE3-40 and ^{3}H-PiB	*−0.5097 (0.0047)*	0.0631 (0.7452)
Aβ forms and ^{3}H-PiB to CAA severity	***Spearman r (P* value)**	***Spearman r (P* value)**
Aβ42 and CAA severity	*0.4088 (0.0164)*	−0.2090 (0.2766)
Aβ40 and CAA severity	0.7786 (<0.0001)	*0.5397 (0.0017)*
AβNpE3-42 and CAA severity	*−0.0092 (0.9608)*	*−0.4759 (0.0079)*
AβNpE3-40 and CAA severity	*0.7672 (<0.0001)*	*0.3273 (0.0474)*
Aβ42/Aβ40 and CAA severity	*−0.7739 (<0.0001)*	*−0.5134 (0.0062)*
AβNpE3-42/AβNpE3-40 and CAA severity	*−0.8394 (<0.0001)*	*−0.4108 (0.0269)*
^{3}H-PiB and CAA severity	*0.3391 (0.0498)*	−0.0419 (0.8141)

For each correlation analysis, data from the frontal cortex and the precuneus were combined. The Spearman rank order correlation test was used to examine associations among variables. Associations meeting criteria for significance (P < 0.05) are italicized.

of Aβ deposits in DS brains when compared to brains of older sporadic AD cases with end-stage pathology. Different proportions of pyroglutamate-modified Aβ and unmodified Aβ in DS compared to preclinical (pathological aging) and clinical AD might affect their detection by amyloid PET. This could have influenced the findings of a longitudinal ^{11}C-PiB PET study which reported slower progression of the frontal cortex and precuneus amyloid pathology in nondemented young adults with DS (mean age 37 years) when compared to nondemented elderly (mean age 73 years; Tudorascu et al., 2019). In contrast to our findings, Hosoda and colleagues reported higher levels of AβNpE3-42 compared to unmodified Aβ1–42 in their DS group (Hosoda et al., 1998). The small number of DS cases and large individual variability of pyroglutamate-modified Aβ levels in the latter report make it difficult to explain this discrepancy.

The preponderance of Aβ40 levels in unmodified and pyroglutamate-modified forms in our DS group appears to be influenced by greater severity of CAA, despite comparable levels of mature amyloid plaques (frequent neocortical neuritic plaques) in the DS and AD groups. This is supported by our observations that levels of Aβ40 and AβNpE3-40 forms (and ratios of Aβ42/Aβ40 and AβNpE3-42/AβNpE3-40) were associated strongly with CAA severity in the DS group, while in the AD group these associations were much weaker. Previous studies reported that CAA is a significant contributor to the neuropathology of DS and is observed more frequently in DS adults over 45–50 years of age than in people with sporadic AD and normal elderly controls (Vinters, 1987; Wilcock et al., 2016; Carmona-Iragui et al., 2017; Head et al., 2017; Davidson et al., 2018). Our results are also consistent with studies reporting that Aβ40 is the primary constituent of vascular amyloid in AD and DS (Miller et al., 1993; Iwatsubo et al., 1995; Harigaya et al., 2000; Guntert et al., 2006; Mann et al., 2018; Gkanatsiou et al., 2019).

We observed that DS and late-stage AD groups had similar levels of ^{3}H-PiB binding in the frontal cortex and in the precuneus. Although DS cases had higher levels of Aβ forms ending at carboxy terminus amino acid 40 and greater severity of CAA, which correlated strongly with greater ^{3}H-PiB binding, the lack of differences in ^{3}H-PiB binding between DS and AD groups appears to be influenced more by these two groups having similar levels of unmodified and pyroglutamate-modified Aβ42 forms. This is in agreement with observations from PiB PET imaging-autopsy studies of AD and *in vitro* analyses of synthetic Aβ that PiB binding is influenced primarily by the Aβ42 form (Ikonomovic et al., 2008, 2020; Yamin and Teplow, 2017) which was reported as the initial and dominant Aβ form in amyloid plaques in AD and DS (Miller et al., 1993; Iwatsubo et al., 1994, 1995, 1996; Lemere et al., 1996). Interestingly, the strongest correlate of (higher) ^{3}H-PiB binding was the (lower) ratio of insoluble AβNpE3-42/AβNpE3-40 in DS, but not in the AD group. However, higher levels of insoluble AβNpE3-40 and Aβ40, as well as greater severity of CAA, do not appear to be the main determinants of ^{3}H-PiB binding levels in DS cases when the overall parenchymal plaque pathology burden is high.

Several potential limitations should be considered in the current study. The commercial ELISA kits we used for the detection of unmodified Aβ forms have been widely applied and reported in published studies. The specificity of their *N*-terminus (detection) antibody is defined in the range of Aβ amino acids 1–16, and this overlaps the range recognized by the well-characterized monoclonal IgG clone 6E10 (Kim et al., 1990). Thus, we cannot be confident that these kits measure

exclusively the intact "full-length" Aβ1–40 and Aβ1–42 forms, because theoretically, they could detect some Aβ forms truncated at the proximal portion of the *N*-terminus. However, *N*-terminus truncated and pyroglutamate-modified forms of Aβ likely undergo additional molecular and conformational modifications and this could interfere with the binding of *N*-terminus-directed antibodies to epitopes that overlap, or are near to, the pyroglutamate modification. Secondly, consistent with brain tissue sampling in a previous analysis of unmodified and pyroglutamate-modified Aβ concentrations in DS and AD (Hosoda et al., 1998), cortical samples in our study were stripped of the leptomeningeal vessels but they included intraparenchymal vasculature, so the ELISA and ^{3}H-PiB binding assays measured insoluble Aβ from the combined pool of amyloid plaques as well as capillary and arterial CAA. This approach is consistent with ^{11}C-PiB PET and related amyloid PET radioligands lacking selectivity to distinguish Aβ plaques from CAA (Bacskai et al., 2007; Johnson et al., 2007; Lockhart et al., 2007; Dierksen et al., 2010; Ly et al., 2010; Sabbagh et al., 2011; Ducharme et al., 2013; Murray et al., 2015; Seo et al., 2017; Charidimou et al., 2018; Planton et al., 2020). Further studies will require biochemical analyses of unmodified and pyroglutamate-modified Aβ forms and ^{3}H-PiB binding in isolated microvessels compared to vessel-free brain parenchyma extracts from DS brains as has been done previously in samples from AD brains (Roher et al., 1993; Kuo et al., 1997; Bourassa et al., 2019) as well as isolated plaque cores in both diagnostic groups to extend previous studies (Allsop et al., 1986; Roher et al., 1993). Lastly, as in most brain banks, the frozen tissue samples for biochemical assays and fixed tissue samples for neuropathological workup were not from the same hemisphere. We assumed, in this study, that neuropathological findings from one hemisphere informed us about the overall brain pathology including the opposite hemisphere from which our samples for ELISA and ^{3}H-PiB binding assays were obtained.

In summary, our study demonstrates that compared to late-stage AD cases, older adults with DS have similar levels of ^{3}H-PiB binding in the frontal cortex and in the precuneus. This is consistent with the observation that both groups had frequent neocortical neuritic plaques and similar levels of Aβ42 and AβNpE3-42 forms in these brain regions. The DS group had more severe CAA pathology and significantly higher levels of Aβ40 and AβNpE3-40 forms in the same cortical regions, however, this was not the key determinant of PiB binding. The presence of CAA is significant because it can impact clinical presentation. For example, CAA independently affects cognition, and when present in conjunction with AD pathology it can result in more severe cognitive impairment (Pfeifer et al., 2002). Interestingly, while the cerebrovascular disease is considered a "second hit" that contributes to the clinical manifestation of AD (Provenzano et al., 2013), DS individuals may have a predisposition that protects against cardiovascular risk factors (Murdoch et al., 1977; Pucci et al., 2016; Lott and Head, 2019), but this protection may be offset by the development of severe CAA pathology in the DS brain. PiB binds to fibrillar Aβ in both parenchymal and vascular (CAA) deposits, and these two pathologies cannot be distinguished unequivocally on amyloid PET using PiB or related amyloid-binding radioligands (Bacskai et al., 2007; Lockhart et al., 2007; Dierksen et al., 2010; Sabbagh et al., 2011; Ducharme et al., 2013). Instead, the presence of CAA in the clinical setting is suspected only if there is a predominance of occipital signal on amyloid PET scans (Johnson et al., 2007; Greenberg et al., 2008; Seo et al., 2017) and higher frequencies of microbleeds, hemorrhagic lesions, or ischemic lesions detected by MR imaging (Dierksen et al., 2010; Ly et al., 2010; Viswanathan and Greenberg, 2011; Yamada, 2015). PET radiotracers selective for CAA are still under development (Abrahamson et al., 2021) and will be critical to incorporate into the neuroimaging biomarker panel for DS, to monitor Aβ deposition in the cerebral vasculature relative to PET measures of total (parenchymal and vascular) amyloid.

DATA AVAILABILITY STATEMENT

The datasets generated in the current study are available from the corresponding author on reasonable request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Institutional Review Board and the Committee for Oversight of Research and Clinical Training Involving Decedents, University of Pittsburgh and University of California, Irvine. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

EA, VV, BH, IL, EH, and MI contributed to the design and implementation of the research. VP, TR, EA, VV, and MI contributed to the analysis of the results. All authors contributed to the writing of the manuscript.

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IntelliCage Automated Behavioral Phenotyping Reveals Behavior Deficits in the 3xTg-AD Mouse Model of Alzheimer's Disease Associated With Brain Weight

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Transgenic rodent models of Alzheimer's disease (AD) were designed to study mechanisms of pathogenesis and connect these mechanisms with cognitive decline. Measurements of cognition in rodents can be confounded, however, by human handling and interaction; the IntelliCage was created to circumvent these issues while measuring various facets of cognition in a social environment with water consumption as the primary motivator for task completion. Here, for the first time, we examined the behavioral performance of 3xTg-AD mice in the IntelliCage. Seven- to 9-month-old female 3xTg-AD and non-transgenic (NonTg) mice were tested for 29 days in the IntelliCage to measure prefrontal cortical and hippocampal function. We found that a higher percentage of NonTg mice (86.96%) were able to successfully complete the training (adaptation) phases compared to their 3xTg-AD (57.14%) counterparts. Furthermore, the 3xTg-AD mice showed impairments in attention and working memory. Interestingly, we found that differences in body and brain weight between NonTg and 3xTg-AD mice were associated with whether mice were able to complete the IntelliCage tasks. 3xTg-AD mice that completed IntelliCage tasks had lower cortical insoluble amyloid-β_{40} fractions than their 3xTg-AD counterparts who failed to complete the tasks. Collectively, these results demonstrate deficits in cognition in the 3xTg-AD mouse and inform scientists of important factors to consider when testing this transgenic model in the IntelliCage.

Keywords: IntelliCage, 3xTg-AD, brain weight, cognition, amyloidosis

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INTRODUCTION

Alzheimer's disease (AD) is the most common cause of dementia and is a top health concern globally; in the US alone, it is projected to rise from 5 million cases currently to ∼15 million by 2050, with national costs rising as high as $1.1 trillion (Alzheimer's association report, 2021). Clinically, AD presents with cognitive impairment including deficits in new memory development, loss of long-term memories that worsen as the disease progresses, and a severe loss of general intellectual ability coinciding with dementia (Honjo et al., 2012; Bateman, 2015). Key neuropathological

features of AD include extracellular plaques of the amyloid-β (Aβ) peptide, neurofibrillary tangles (NFTs)—intraneuronal tangles of hyperphosphorylated tau—and synaptic and neural loss (Honjo et al., 2012; Bakota and Brandt, 2016; Lane et al., 2018). Therapeutic options remain extremely limited for advanced AD, highlighting the need for preclinical research into molecular events preceding Aβ and NFT pathology prior to dementia onset.

Aβ plaques observed in AD are derived from the Aβ peptide, which ranges from 36 to 43 amino acids in length. $Aβ_{40}$ and $Aβ_{42}$ are the most abundant Aβ species observed in AD (O'Brien and Wong, 2011; Sadigh-Eteghad et al., 2015). The shift from soluble to insoluble forms of $Aβ_{40}$ and $Aβ_{42}$ has been identified as what may distinguish dementia pathology from normal aging (Wang et al., 1999). It has been shown that $Aβ_{40}$ plays a mechanistic role in the onset and/or progression of AD (Wang et al., 1999), and while insoluble $Aβ_{40}$ is more prevalent than $Aβ_{42}$, insoluble $Aβ_{42}$ is more prone to aggregation and toxicity (O'Brien and Wong, 2011). The AD brain is also characterized by tau phosphorylation and NFTs (Braak et al., 2006). Numerous reports have highlighted that phosphorylation at specific tau sites, in particular, Ser202/Thr205 (AT8) and Thr214/Ser212 (AT100), is highly associated with increased intraneuronal filaments and neurodegeneration (Allen et al., 2002; Augustinack et al., 2002). Braak Staging, a well-established method that scores the accumulation of phospho-tau, is based on AT8 antibody staining (Braak and Braak, 1991; Braak et al., 2006); researchers have also identified the sequential appearance of specific tau phospho-dependent epitopes, revealing that AT100 phosphorylation appears after AT8 in human AD post-mortem brain tissue (Luna-Muñoz et al., 2007).

AD rodent models remain a key tool for unraveling pathogenic mechanisms and for the development of preclinical therapeutic options; 205 mouse models possessing many of the aspects of human AD are in use worldwide as of 2021 (Alz Forum, 2021). These models are developed by incorporating key human transgenes associated with familial AD into the mouse genome-mice do not spontaneously develop AD (Borchelt et al., 1997; Jankowsky et al., 2004; Reiserer et al., 2007; Hall and Roberson, 2012; LaFerla and Green, 2012). One of the most widely-used AD models that recapitulates hallmark Aβ and NFT pathologies is the 3xTg-AD mouse model, developed in 2003 (Oddo et al., 2003). This model incorporates the APP human Swedish mutation, a presenilin knock-in mutation, and a human P301L mutation. Aβ deposits are present by 6 months of age in the frontal cortex and become more extensive by 12 months of age in this model (Oddo et al., 2003; Sterniczuk et al., 2010). AT8 tau phosphorylation is widespread in the hippocampus by 6 months of age, while AT100 tau phosphorylation is more widespread in the hippocampus by 12 months (Oh et al., 2010; Parachikova et al., 2010). Spatial cognition and memory deficits have been documented as early as 6 months and become more pronounced by 12 months of age in the Morris water maze (MWM) spatial cognition task (Parachikova et al., 2010; Roda et al., 2020). Notably, reports have shown that brain weight in 15-month-old 3xTg-AD mice is reduced compared to NonTgs (Di Benedetto et al., 2019). Brain size is highly relevant and underexplored in the 3xTg-AD mice, as studies highlight that brain size may be associated with poorer cognitive function (Perepelkina et al., 2020).

Behavioral tasks have been developed to evaluate cognition in rodents, including in AD mouse models. However, these behavioral tasks rely on the daily handling of rodents while being transported to the testing apparatuses and during testing itself. Such testing protocols also contain other caveats, including experimental variability due to environmental factors, animal isolation, unnatural incentives to participate (e.g., water escape), and human errors in scoring. In 2000, Dr. Hans-Peter Lipp developed the IntelliCage, which helps overcome these factors by allowing rodents to be tested in their natural social environment with minimal human intervention (Dell'Omo et al., 2000; Lipp, 2005; Lipp et al., 2005). The IntelliCage is a fully-automated system allowing animals to engage in a wide variety of experimental tasks, using access to water as their incentive to participate, and has since been used in over 150 studies (Lipp et al., 2005; Lipp, 2005; Masuda et al., 2018; Kiryk et al., 2020; Mifflin et al., 2021). Animals can be assessed for exploratory behavior, water consumption patterns, spatial learning, behavioral flexibility, attention, impulsivity, and working and contextual memory within a single cage. While APP/KI and APP/PS1 mouse models of amyloidosis have been tested in the IntelliCage, to date, the 3xTg-AD model has not been evaluated in this automated behavioral phenotyping apparatus (Ryan et al., 2013; Lee et al., 2015; Masuda et al., 2016; Mifflin et al., 2021).

The goal of the present work was to test the 3xTg-AD mouse for the first time in the IntelliCage and determine if cognitive deficits can be detected. Additionally, we aimed to determine whether AD-like pathologies in 3xTg-AD mice-brain size, body weight, Aβ, and pathological tau-are associated with behavioral results from various phases of IntelliCage testing. Given previous reports relating brain size, Aβ, and pathological tau with cognition (Sterniczuk et al., 2010; Perepelkina et al., 2020), we hypothesized that increased presentation of these pathologies would be associated with deficits in the IntelliCage tasks.

MATERIALS AND METHODS

Animals

3xTg-AD mice were generated on a C57BL6/129Svj hybrid background as previously described (Oddo et al., 2003; Velazquez et al., 2019c). Since 3xTg-AD mice are homozygous for mutations in the APP, PS1, and MAPT genes, colonies are maintained by breeding homozygous 3xTg-AD mice to each other. Notably, 3xTg-AD males show large neuropathological variability, even between littermates, while females do not show such variability. Therefore, as in most recent studies using the 3xTg-AD mouse, we only included female mice. All protocols were approved by the Institutional Animal Care and Use Committee of Arizona State University and conform to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Mice were group housed

(4 to 5 mice per cage) prior to being introduced into the IntelliCage. At 7–9 months of age, prior to IntelliCage testing, a radiofrequency identification transponder chip (RFID; Standard Microchip T-VA, DataMars, Switzerland and Troven, USA) was subcutaneously implanted into the dorso-cervical region under isoflurane inhalation anesthesia as previously described (Mifflin et al., 2021). The RFID chip allows for the identification of a mouse when it enters a corner of the IntelliCage system. Mice were allowed 1 week to recover and were then introduced into the IntelliCage.

Female 3xTg-AD (n = 21) and C57BL6/129Svj (n = 23; herein referred to as NonTg) mice were placed in the IntelliCage for assessment across a variety of tasks tapping hippocampal and prefrontal cortical function (Ajonijebu et al., 2018; Voikar et al., 2018; Kiryk et al., 2020). IntelliCage testing took a total of 29 days from adaptation to the place avoidance retention phase. Each IntelliCage holds up to 16 mice, and our lab currently has three cages allowing us to test all of the mice in one cohort. We introduced the following number of mice to each cage: cage one n = 8 3xTg-AD and n = 8 NonTg, cage two n = 7 3xTg-AD and n = 8 NonTg, and cage three n = 6 3xTg-AD and n = 7 NonTg mice. Mice were subsequently euthanized at 8–10 months of age, and tissue was extracted and prepared for ELISAs and western blot analysis.

Automated IntelliCage Testing

The IntelliCage was used to evaluate water drinking, exploratory behavior, spatial learning, reference memory, behavioral flexibility, attention, and contextual memory (Masuda et al., 2016, 2018; Kiryk et al., 2020; Mifflin et al., 2021). The testing apparatus (39 cm × 58 cm × 21 cm) contains four corner chambers accessible through an antenna-equipped open tunnel. A computer management system is used to regulate water access *via* two individual doors in each corner. A scanner, located at each corner entrance, registers each animal's entrance by scanning the individual RFID; an animal's entire body must enter the corner to register the RFID and for a visit to be counted. Nosepokes and licks are detected by sensors on the nose port and waterspout, respectively. Mice were fed *ad libitum* during the entire duration in the IntelliCage with standard mouse chow, and lights were on in the behavior room from 06:00–20:00. A video camera was placed outside the IntelliCage and recorded the entire testing session (24 h/7 days a week). The sequence of experimental behavioral tasks in the IntelliCage was as follows: (1) Adaptation, consisting of free adaptation, door adaptation, and nosepoke adaptation phases; (2) Place preference and reversal; (3) Serial reaction time; and (4) Place avoidance. Any animal that failed to consume water in a 24-h period was removed from the IntelliCage and placed in a standard cage for 7 h to avoid severe dehydration. If these animals were re-introduced into the IntelliCage and again failed to consume water, they were removed from the experiment.

Data were extracted using the TSE IntelliCagePlus Analyzer software, from which we exported the data into multiple tab-delimited text files. Then, using a Python script, the text files were converted into a single SQLite3 database file. Using this file as input, several Python scripts were then used to query the database using SQL to extract the number of visits, nosepokes and licks, in addition the visits with at least one nosepoke to each corner, visits with at least one lick to each corner, delay to first visit, delay to the first nosepoke, and delay to the first drink for each animal. The same script also sliced the data into 24-h periods and separated the data into Excel spreadsheets with the data for each day. For each task, the dependent variable calculated is described below.

(1) Adaptation Phases

- During the first 3 days of the adaptation phase (free adaptation) all the doors were open allowing free access to the water bottles, thereby acclimating mice to the new environment. During the next 3 days (door adaptation), the doors to the water bottles were closed but opened for any visit into the corner. The following were calculated for the free adaptation phase:
- Total visits (to measure exploratory and water-seeking behavior)
- Total licks (to measure water consumption)

For the last 3 days (nosepoke adaptation), doors were closed and could be opened with a nosepoke in a corner, thereby training animals to nosepoke to retrieve water. In addition to total visits and total licks, the following were calculated:

- Number of visits with $\geq$1 nosepoke (indicating adaptation/learning)
- Number of visits with $\geq$1 lick over total visits (indicates the number of visits due to water-seeking motivation instead of exploratory behavior)

(2) Place Preference and Reversal

- During the place preference phase, water was accessible in only one of the four corners for each of the mice. The correct corner for each mouse was chosen based on their previous visit habits, selecting among the least-visited corners to eliminate preferential corner bias. For the first 6 days, water was available only in the selected reward corner (place preference). For the last 6 days, water was available only in the opposite corner (reversal). To prevent overcrowding of the corners and learning by imitation, the selected reward corners were balanced by the number of mice and genotype, limiting the number to four per corner and 50–50 proportion of 3xTg-AD and NonTg genotypes. In addition to measuring total visits and licks per day, calculations for this task were as follows:
- % correct = (number of visits to correct corner)/(total corner visits)
- Correct visit with a lick = (number of visits to correct corner with nosepoke and $\geq$1 lick)/(total visits to correct corner)

(3) Serial Reaction Time (SRT) Attention task

- During the 3 days of the SRT task, when an animal entered an assigned corner (the correct corner from the prior place preference reversal task), the first nosepoke for a visit

initiated a trial. A 2 s pre-cue delay was imposed prior to the illumination of a green LED, requiring mice to learn to wait for the LED. The green LED was turned on for 7 s, requiring the animal to nosepoke within this time frame to count as a correct response, resulting in the door opening and allowing access to water. A nosepoke during the 2 s pre-cue delay was counted as a premature response and is a measure of impulsivity. If the animal failed to nosepoke during the 7 s time frame, the LED turned off and the trial would reset, requiring the animal to restart the trial. In addition to measuring total visits and licks per day, calculations for this task were as follows:

- % initiated trials = (number of correct visits with a first nosepoke)/(total visits)
- % correct = (number of correct visits with nosepoke and lick)/(total visits)
- % incorrect = (number of correct visits without a nosepoke and lick)/(total visits)
- % premature = (number of correct visits with a response during the 2 s pre-cue delay)/(number of initiated trials)
- Reaction time = time (s) to extinguish LED during correct visits with a nosepoke

(4) Place Avoidance

- The place avoidance tasks included both training and probe trials. For day 1, 24-h training trial (learned avoidance), nosepoking in the reward corner administered an aversive air puff (∼0.8 bar, 1 s air-puff). The doors in all corners remained closed and water was not available during the learned avoidance phase. In addition to measuring total visits per day, we also analyzed the number of corner visits with nosepokes at the air puff corner to assess working memory errors. After the 24-h training trial, the mice were moved to their standard home cages for a 24-h delay with water *ad libitum*. After the delay, the mice were reintroduced to the IntelliCage for 3 days with water available at all four corners and the air puff stimulus removed to assess retention and extinction. The data for retention and extinction was quantified as the % correct visits with nosepokes over total visits for each day.

Brain Tissue Processing, ELISA, Western Blots, and Immunohistochemistry

At the completion of IntelliCage testing, mice body weights were recorded, and their brain was extracted and weighed. One hemisphere had the hippocampus and cortex dissected out and flash-frozen while the contralateral hemisphere was fixed in a glass vial of 4% paraformaldehyde for 48 h and then transferred into 0.02% sodium azide in phosphate-buffered saline until sectioning. 50-μm-thick free-floating sections were subsequently obtained using a vibratome and used for histology. Flash-frozen tissue was homogenized in a T-PER tissue protein extraction reagent supplemented with protease (Roche Applied Science, IN, USA) and phosphatase inhibitors (Millipore, MA, USA). The homogenized tissues were centrifuged at 4°C for 30 min, and the supernatant (soluble fraction) was stored at −80°C. We then homogenized the pellet in 70% formic acid followed by centrifuging at 4°C for 30 min. Hippocampal and cortical soluble and insoluble fractions of $A\beta_{40}$ and $A\beta_{42}$ were detected using the commercially available ELISA kits (Invitrogen-ThermoFisher Scientific) as previously described (Velazquez et al., 2019a,b). Western blots were performed under reducing conditions as we previously detailed (Velazquez et al., 2016, 2019a). Quantitative analyses of the western blots were obtained by normalizing the intensity of the protein of interest with its own loading control β-actin, within each blot. Licor Image Studio software was used to quantify the intensity of the bands of interest. The experimenter was blinded to the group allocations.

Immunohistochemistry for AT8 was performed as we previously described (Dave et al., 2021). Images from three sections per mouse including dorsal, medial, and ventral hippocampus were taken with a Zeiss Axio Imager A1 using a 40× objective. Images were photomerged to rebuild the image, and AT8+ cell number was obtained using ImageJ. The experimenter was blinded to the group allocation.

Antibodies

All the antibodies used in this study have been validated by the manufacturer for use in mouse tissue. The following antibodies were purchased from Thermo Fisher; AT8 (1:500 dilution, catalog #MN1020); AT100 (1:500 dilution, catalog #MN1060); β-actin (1:10,000 dilution, catalog #PA1-16889). The following antibody was purchased from MilliporeSigma, 6E10 (FL-APP 1:1,000 dilution, catalog #MAB1560).

Statistical Analyses

ANOVA was used to examine the various IntelliCage data with repeated measures when applicable using StatView 5.0.1 (SAS Institute) and GraphPad Prism 8.1.2. Data that included repeated dependent measures were first assessed for sphericity using Mauchly's tests, and no violations were found necessitating corrections. Bonferroni's corrected *post hoc* tests were performed when a significant interaction was observed. Student's unpaired *t*-tests were employed for comparison of 3xTg-AD mice when appropriate. Examination of descriptive statistics revealed no other violations of any assumptions that required the use of statistical tests other than the ones used. Significance was set at $p < 0.05$.

RESULTS

A Higher Percentage of 3xTg-AD Mice Failed to Complete the Adaptation Phases of the IntelliCage

A total of 21 3xTg-AD mice and 23 NonTg mice were placed into the IntelliCage and started on the adaptation phases (**Figures 1A–C**). We found that a higher percentage of NonTg mice (86.96%) were able to make it past the adaptation phases compared to the 3xTg-AD counterparts (57.14%; **Table 1**); failure to pass the free adaptation phase was due to animals failing to drink. We next analyzed data for animals able to pass the free adaptation phase. During the 3 days of free adaptation, when all corners were accessible for water consumption (**Figure 2A**),

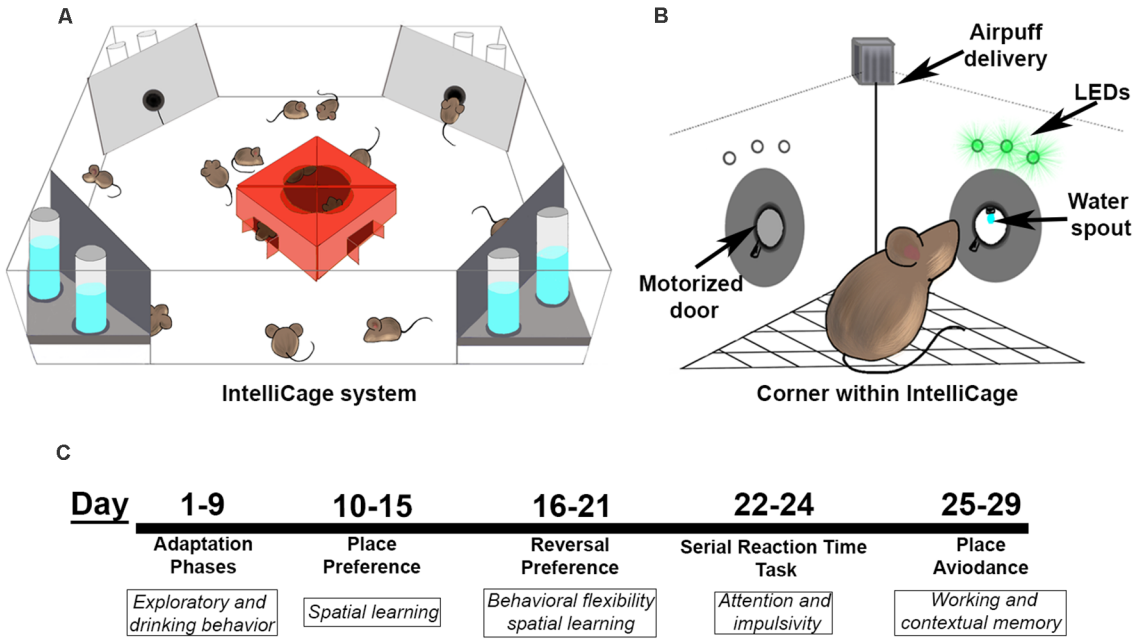

FIGURE 1 | The IntelliCage and testing paradigm. **(A)** The IntelliCage accommodates up to 16 mice at a time and contains four corners that are programmed to allow water access based on nose port responses. **(B)** Each corner in the IntelliCage is equipped with two nose ports with doors, LEDs above each nose port, an air delivery nozzle, and access to water with a lickometer to detect drinking behavior. **(C)** Behavioral battery timeline for the IntelliCage study. Mice started testing at a range of 7–9 months of age.

we found a significant main effect of the day ($F_{(2,60)}$ = 10.013, $p < 0.001$; **Figure 2B**), where total corner visits decreased across days. We also found a significant genotype by day interaction ($F_{(1,60)}$ = 6.283, $p < 0.01$; **Figure 2B**); *post hoc* analysis revealed that NonTg mice made more corner visits than the 3xTg-AD mice on day 1 ($p < 0.05$). No differences in total licks were found during the free adaptation phase (**Figure 2**C), indicating that water consumption did not vary. Next, we analyzed data for the nosepoke adaptation phase, where animals had to nosepoke to receive water. We found no significant differences in total visits, total visits with a nosepoke, or total licks during the nosepoke adaptation phase (**Figures 2D–F**). To determine whether corner visits were due to exploration or water consumption, we analyzed the number of visits with ≥1 lick over total visits. We found a significant main effect of genotype ($F_{(1,60)}$ = 6.869, $p < 0.05$; **Figure 2G**), where the 3xTg-AD entered corners to drink more frequently than the NonTg mice, despite both groups consuming the same volume of water (i.e., total licks). We also found a significant genotype by day interaction ($F_{(2,60)}$ = 4.911, $p < 0.05$; **Figure 2G**). *Post hoc* analysis revealed that the 3xTg-AD entered corners to drink significantly more often than the NonTg mice on day 2 ($p < 0.01$) and 3 ($p < 0.01$). Collectively, these results indicate that a higher percentage of 3xTg-AD mice were not capable of learning to drink during the adaptation phases of the IntelliCage; those 3xTg-AD mice that did make it to the nosepoke adaptation phase entered corners frequently with the purpose of consuming water, while their NonTg counterparts' motivation to enter corners varied between both exploration and water consumption.

TABLE 1 | A higher percentage of NonTg mice were able to learn to enter a corner and drink from the waterspout compared to 3xTg-AD mice, resulting in a higher exclusion of 3xTg-AD mice from the subsequent IntelliCage tasks.

Genotype	Total start *n*	Total passed adaptation phase	% *n* passed adaptation
NonTg	23	20	86.96%
3xTg-AD	21	12	57.14%

Nine 3xTg-AD and one NonTg mice failed to drink during Day 1–3 of adaptation. Two NonTg mice failed to drink during the door-adaption days (Day 4–7).

3xTg-AD Mice Performed Similarly to NonTg Mice in Learned Place Preference but Later Performed Better Than NonTg Mice in the Reversal Phase of the IntelliCage

During the learned place preference phase, animals were assigned to and only granted access to water from one corner (**Figure 3A**). Mice can use external environment cues to locate their correct corner, thereby assessing spatial learning (Ryan et al., 2013; Lee et al., 2015; Kiryk et al., 2020). We found no significant differences in total visits (**Figure 3B**) and total licks (**Figure 3**C), illustrating that both genotypes visited corners and drank equal amounts of water during this phase. We found a significant main effect of day for % correct in the place preference phase $F_{(1,140)}$ = 19.063, $p < 0.0001$; **Figure 3D**), illustrating that performance improves across the 6 days for both genotypes. Lastly, to determine if animals visited the correct corner to drink or explore, we examined the visits to the correct corner

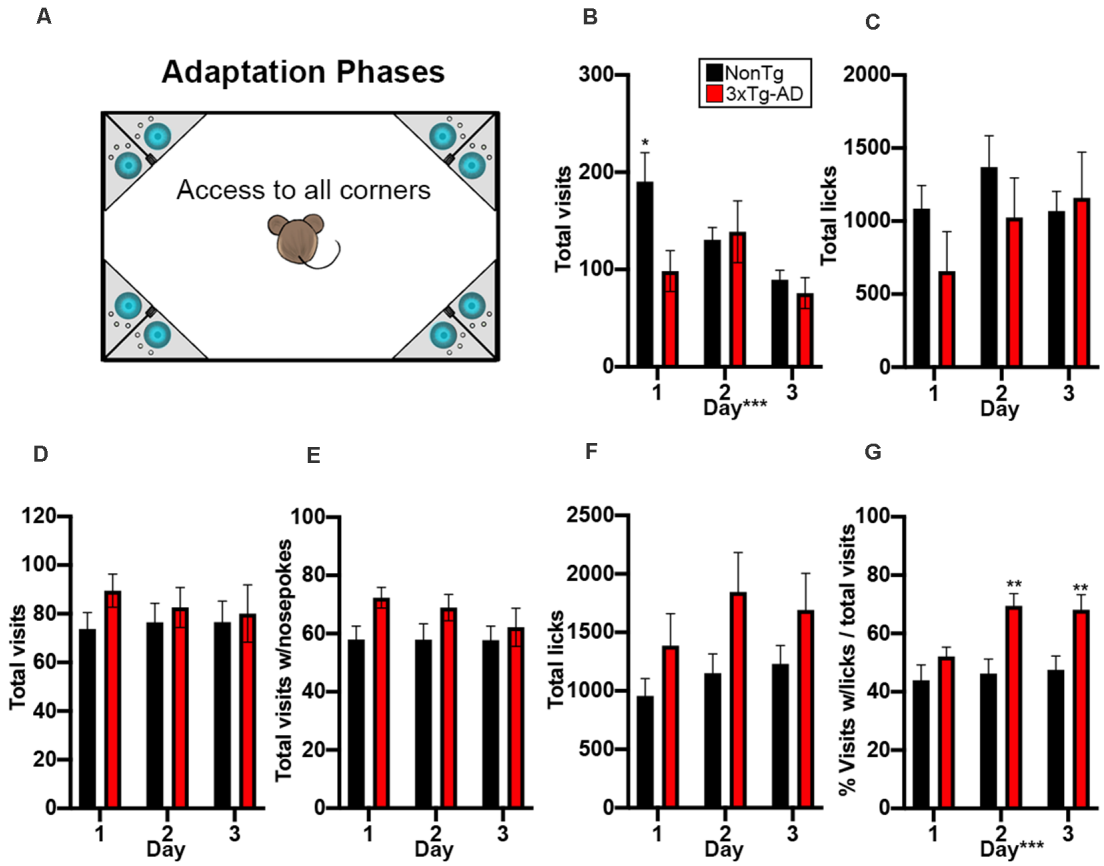

FIGURE 2 | NonTg mice explore more and drink less than 3xTg-AD mice in the adaptation phases. **(A)** During the 3-day adaptation phase, animals freely entered any of the four corners and could access water: cage not drawn to scale. **(B)** We found that NonTg mice made more corner visits on day 1 compared to 3xTg-AD mice ($p < 0.05$). We also found that the number of visits decreased from day 1–3 ($p < 0.001$). **(C)** No differences detected in total licks. In the 3-day nosepoke adaptation phase, animals must learn to nosepoke to access water. **(D–F)** No significant differences were detected in total visits, total visits with a nosepoke, and total licks. **(G)** We found that 3xTg-AD mice made more total visits to lick than NonTg mice on days 2 and 3 of nosepoke adaptation, illustrating increased motivation to enter a corner and nosepoke to drink. Data are presented as means ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

with ≥1 lick. We found no significant differences, suggesting that in this phase, both genotypes' motivation to enter the correct corner was at least in part due to water-seeking (**Figure 3E**).

Next, animals were assessed in the place preference reversal phase (**Figure 3F**), where the correct corner is opposite to that of the first phase of place preference. We found no significant differences in total visits between the NonTg and 3xTg-AD mice for this phase (**Figure 3G**). We did find a significant main effect of day for total licks ($F_{(1,140)} = 4.450$, $p < 0.001$, **Figure 3H**), illustrating increasing licking across the 6 days. For % correct, we found a significant main effect of day ($F_{(1,140)} = 33.100$, $p < 0.0001$; **Figure 3I**), illustrating learning across the 6 days. Surprisingly, we also found that the 3xTg-AD mice had a higher % correct in reversal preference phase than the NonTg mice ($F_{(1,140)} = 7.767$, $p < 0.01$; **Figure 3I**). When we examined the number of visits to the correct corner with ≥1 lick, we found a significant main effect of genotype ($F_{(1,140)} = 64.107$, $p < 0.0001$; **Figure 3J**), where the 3xTg-AD mice made a higher number of correct corner visits with ≥1 lick than the NonTg mice. We also found a significant main effect of day ($F_{(1,140)} = 6.414$, $p < 0.0001$), illustrating increased correct visits with ≥1 lick across the 6 days. Together, these results suggest that there are no differences in the early phase of preference learning between NonTg and 3xTg-AD mice; however, once animals learn the task, the 3xTg-AD mice made more correct visits to access water than the NonTg mice during place preference reversal. The number of visits to the correct corner with ≥1 lick was higher in 3xTg-AD compared to NonTg, suggesting that later in the place preference tasks, water seeking was the stronger motivator for corner visits among the 3xTg-AD mice while exploration played a dominant motivational role in NonTg mice.

3xTg-AD Mice Showed Impairments in Attention in the Serial Reaction Time (SRT) Tasks

To determine whether animals showed impairments in attention, increased impulsivity, or delayed reaction time, we next tested mice in the SRT attention task (**Figure 4A**). We first analyzed

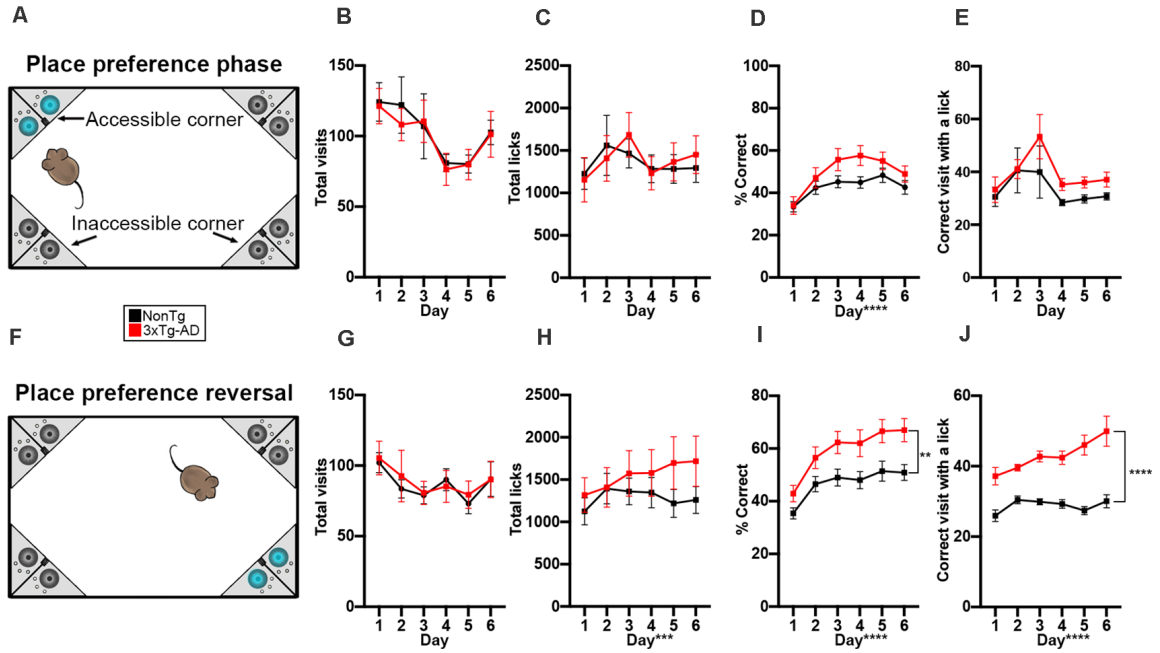

FIGURE 3 | 3xTg-AD mice performed similarly to NonTg mice in learned place preference but later performed better than NonTg mice in the reversal phase of the IntelliCage. **(A)** During the 6 days of the learned place preference phase, animals were assigned to one corner where they could access water. All other corners were counted as incorrect and allowed no access to water; cage not drawn to scale. **(B,C)** We found no significant differences in total visits and licks during the place preference phase. **(D)** We found a significant effect of day for % correct, illustrating learning across the 6 days, but no genotype differences were detected. **(E)** No differences were detected in the correct visits with ≥1 lick. **(F)** During the 6 days of the place preference reversal phase, animals could access water by entering and nosepoking the opposite corner from the corner assigned during the learned place preference phase; cage not drawn to scale. **(G)** No differences in total visits across the 6 days were detected. **(H)** We found that total licks increased across the 6 days ($p < 0.001$). **(I)** We found that 3xTg-AD had a higher % correct than NonTg ($p < 0.01$). Additionally, % correct increased across the 6 days ($p < 0.0001$), illustrating learning. **(J)** We found that 3xTg-AD mice made more visits to the correct corner with ≥1 lick than NonTg mice, illustrating that 3xTg-AD mice were motivated to enter the correct corner to drink. Additionally, correct corner visits with ≥1 lick increased across the 6 days ($p < 0.0001$). Data are presented as means ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

total visits and found a significant genotype by day interaction ($F_{(2,56)} = 5.182$, $p < 0.01$; **Figure 4B**). *Post hoc* analysis revealed that 3xTg-AD made more corner visits than NonTg mice on days 2 ($p < 0.01$) and 3 ($p < 0.01$). We found a main effect of the day for number of initiated trials in the correct corner during SRT, illustrating increased trial initiation per day ($F_{(2,56)} = 4.711$, $p < 0.05$; **Figure 4C**). We also found a main effect of day for total licks ($F_{(2,56)} = 8.671$, $p < 0.001$; **Figure 4D**), where the number of licks increased by the day. For % correct, we found a significant main effect of the day ($F_{(1,56)} = 44.639$, $p < 0.0001$, **Figure 4**E), illustrating learning across SRT testing days. We also found a main effect of genotype ($F_{1, 56)} = 4.751$, $p < 0.05$; **Figure 4E**), where the 3xTg-AD had a significantly lower % correct than the NonTg mice. Consistently, we found a main effect of day for % incorrect visits ($F_{(1,56)} = 44.639$, $p < 0.0001$, **Figure 4F**), where % incorrect went down across the SRT days, further illustrating learning. We also found a main effect of genotype ($F_{(1,56)} = 4.751$, $p < 0.05$) where the 3xTg-AD had a significantly higher % incorrect than NonTg mice. Failure to wait during the 2 s pre-cue delay between trial initiation and cue onset indicates impulsive behavior; when we measured % premature responses, we found a significant main effect of day ($F_{(1,56)} = 11.169$, $p < 0.0001$, **Figure 4G**), indicating that premature responses during the 2 s pre-cue delay went down across the SRT days. Notably, we found no significant genotype differences for premature responses (**Figure 4G**). Lastly, we measured reaction time to extinguish the LED and found no significant differences between the two genotypes (**Figure 4H**). Collectively, these results show attention deficits in the 3xTg-AD mice.

3xTg-AD Mice Showed Impairments in Working Memory During the Place Avoidance Task

In the final phase of the IntelliCage, we tested all mice in a place avoidance learning task to measure both working and contextual memory (**Figure 5A**). During the 24-h period of airpuff exposure, entry into the correct corner from the SRT phase and a nosepoke resulted in an airpuff. We found a significant difference for total visits between the two genotypes ($t_{(28)} = 2.302$, $p < 0.05$, **Figure 5B**), where 3xTg-AD mice made more total visits than the NonTg mice. We also found a significant genotype difference for nosepokes within the airpuff corners ($t_{(28)} = 2.642$, $p < 0.05$, **Figure 5C**), where the 3xTg-AD mice entered the airpuff corner and nosepoked significantly more than the NonTg mice, illustrating deficits in working memory. After the 24-h airpuff

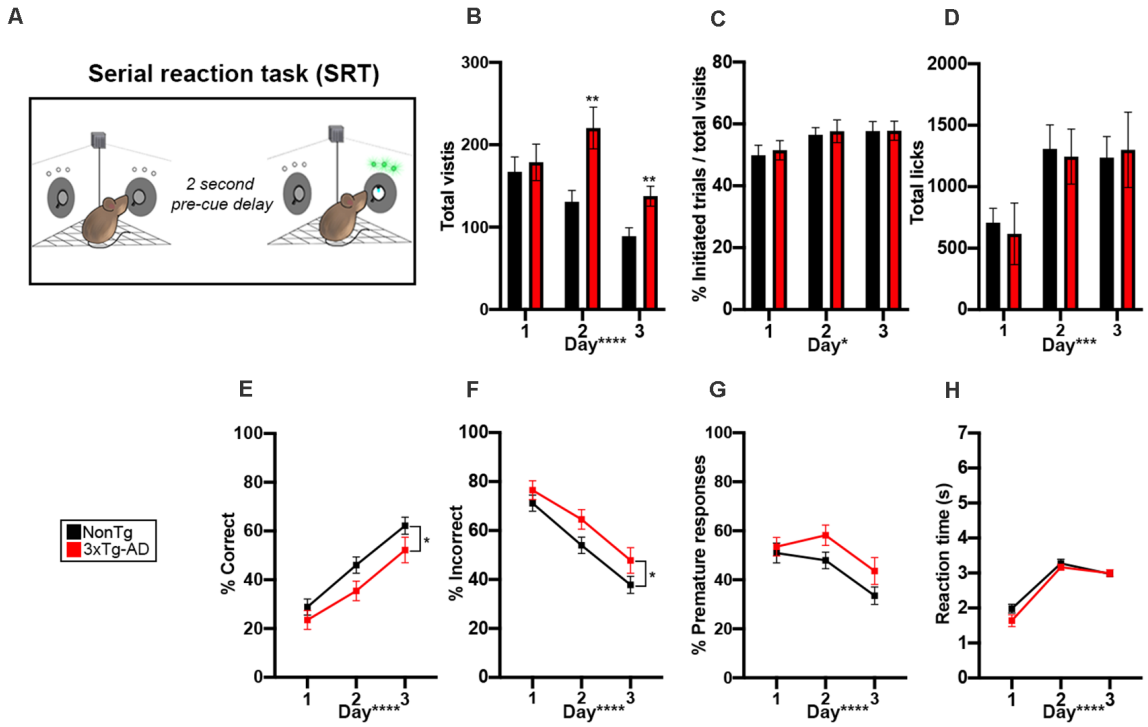

FIGURE 4 | 3xTg-AD mice showed impairments in attention in the Serial reaction time (SRT) attention tasks. **(A)** During the 6 days of the SRT task, animals were required to enter an assigned corner and nosepoke to initiate a trial. Then, a 2-s pre-cue delay was initiated and animals needed to learn to wait for the cue illumination. A green LED illuminated in one of the two noseports and the animal had 7 s to extinguish the LED with a nosepoke. A correct response resulted in access to water, while a premature (during 2-s delay) or incorrect response reset the trial and the animal was required to leave the corner before initiating a new trial; corner not drawn to scale. **(B)** 3xTg-AD mice made more total visits to all corners on day 2 ($p < 0.01$) and 3 ($p < 0.01$) of testing. Total visits went down across the 3 days of testing ($p < 0.0001$). **(C,D)** The number of initiated trials ($p < 0.05$) and total licks ($p < 0.001$) went up across the 3 days of testing. **(E)** % correct increased throughout the testing days ($p < 0.0001$), indicating learning. We found that 3xTg-AD mice had a significantly lower % correct than NonTg counterparts ($p < 0.05$). **(F)** % incorrect decreased throughout the testing days ($p < 0.0001$), indicating learning. We found that 3xTg-AD mice had a significantly higher % incorrect than NonTg counterparts ($p < 0.05$). **(G,H)** % premature responses decreased ($p < 0.0001$) and reaction time to extinguish the LED ($p < 0.0001$) increased across the 3 days. Data are presented as means ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

exposure, mice were removed from the IntelliCage and placed in a standard cage for 24 h. The mice were then returned to the IntelliCage to assess memory and extinction by measuring corner visits with nosepokes to the previously assigned airpuff corner; all corners had water accessible in this phase (**Figure 5D**). We found no significant differences in total visits or in % error with nosepoke for the 3-day retention phase between the 3xTg-AD and NonTg mice (**Figures 5E,F**). In conclusion, the 3xTg-AD mice enter the airpuff corner and nosepoke significantly more illustrating working memory errors but remember the airpuff corner equally to NonTg mice after a 24-h delay.

3xTg-AD Mice Unable to Complete the IntelliCage Adaptation Phases Showed Increased Body Weight and a Reduced Brain to Body Ratio

Upon completion of IntelliCage testing, all mice were weighed and subsequently euthanized, with brains weighed and collected for neuropathological assessment. We first analyzed body, brain, and brain/body weight ratios to determine differences between NonTg ($n = 23$) and 3xTg-AD ($n = 21$). This included all mice, regardless of whether they completed or did not complete the IntelliCage testing (**Table 2**). We found a significant difference in body weight ($t_{(42)} = 2.701$, $p < 0.01$), where the 3xTg-AD mice weighed significantly more than the NonTg mice. Next, we measured brain weight and found a highly significant difference ($t_{(40)} = 10.08$, $p < 0.0001$), where 3xTg-AD mice have a lower brain weight than NonTg counterparts. We then examined the brain/body ratio and found a highly significant difference ($t_{(40)} = 5.293$, $p < 0.0001$), where the 3xTg-AD mice have a lower brain/body ratio than NonTg mice. Next, we examined whether these morphometric disparities persisted when comparing the NonTg ($n = 18$) and 3xTg-AD ($n = 12$) mice who were able to complete the IntelliCage tasks. There were no genotype differences for bodyweight of mice that completed the IntelliCage tasks. We did however find a significant difference for brain weight ($t_{(26)} = 7.140$, $p < 0.0001$), where 3xTg-AD mice had a lower brain weight than the NonTg mice. We also found a significant difference in brain/body ratio ($t_{(26)} = 3.535$, $p < 0.01$), where the 3xTg-AD mice had a significantly

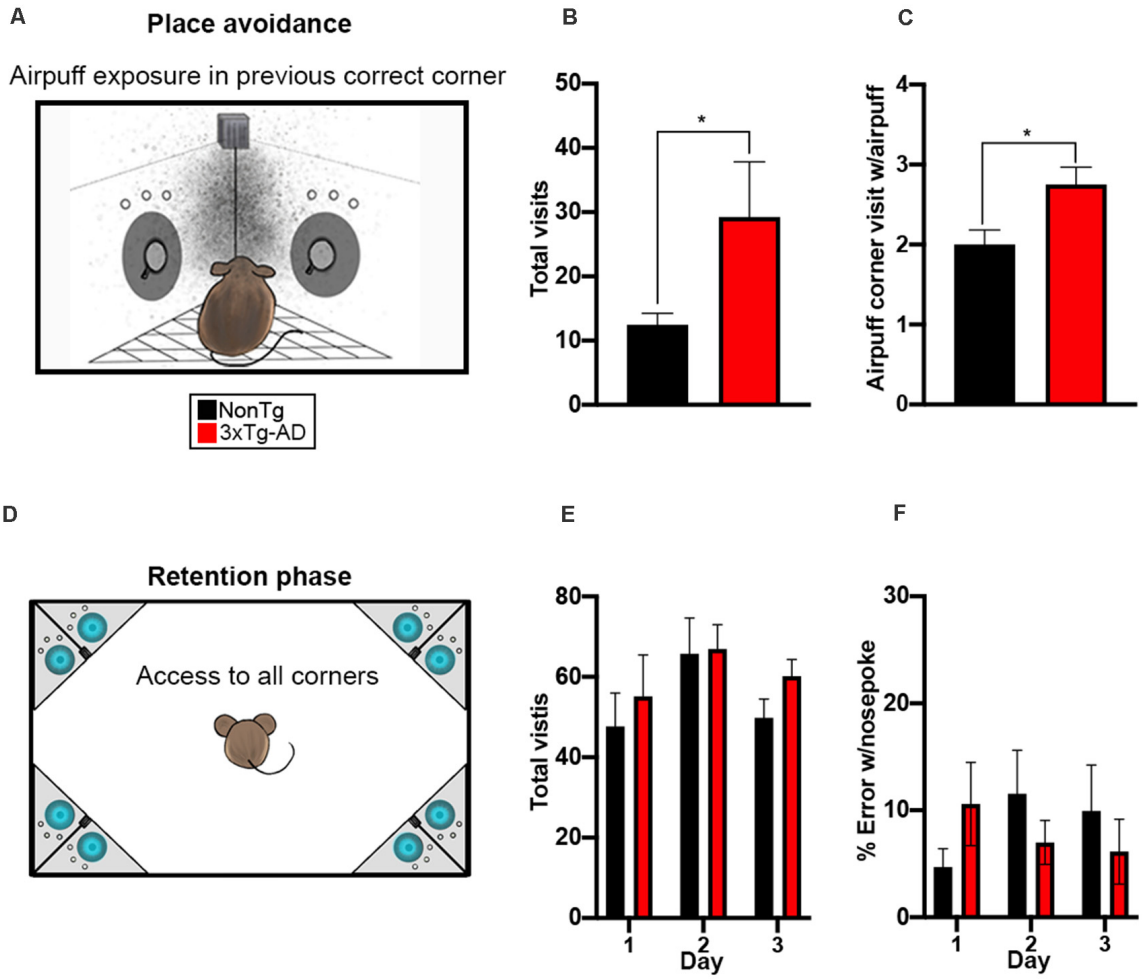

FIGURE 5 | 3xTg-AD mice show impairments in working memory during the place avoidance task. **(A)** For a 24-h period, entry into the assigned corner with a nosepoke resulted in an airpuff (~0.8 bar, 1 s airpuff); cage not drawn to scale. **(B,C)** We found that 3xTg-AD mice made more total visits ($p < 0.05$) and more visits to the airpuff corner with a nosepoke ($p < 0.05$) than the NonTg mice. **(D)** Mice were removed from the IntelliCage after the airpuff exposure and placed in a standard cage for 24 h, then returned to the IntelliCage to assess memory and extinction by measuring corner visits to the previously assigned airpuff corner; cage not drawn to scale. **(E,F)** No significant differences in total visits or % error with a nosepoke (i.e., visiting the airpuff corner from exposure) were detected. Data are presented as means ± SEM. *$p < 0.05$.

lower ratio than NonTg mice. Lastly, we examined body and brain weight differences in 3xTg-AD mice that completed the IntelliCage tasks (complete; $n = 12$) compared to those that could not complete adaptation phases (incomplete; $n = 9$). We found a significant difference in body weight ($t_{(19)} = 2.970$, $p < 0.01$), where 3xTg-AD complete mice weighed less than the 3xTg-AD incomplete mice. There were no significant differences for brain weight between the 3xTg-AD complete and incomplete mice. However, we found a significant difference in brain/body ratio ($t_{(19)} = 3.396$, $p < 0.01$), where 3xTg-AD complete mice had a higher brain/body ratio than the 3xTg-AD incomplete mice. These results show a difference in body and brain weight between NonTg and 3xTg-AD mice and suggest that brain weight and brain/body ratio may be a factor resulting in mice being unable to complete the IntelliCage tasks.

3xTg-AD Mice That Were Unable to Complete the IntelliCage Adaptation Phases Showed Increased Insoluble Aß$_{40}$ Levels and Decreased Insoluble Aß$_{42}$ Levels

To determine whether common AD-like neuropathology markers could help explain why 3xTg-AD mice completed (complete; $n = 12$) or did not complete (incomplete; $n = 9$) IntelliCage tasks, we measured soluble and insoluble fractions of Aβ_{40-42} from hippocampal and cortical samples *via* ELISA. We found no significant differences for both soluble Aβ_{40} and Aβ_{42} levels for the 3xTg-AD complete compared to the 3xTg-AD incomplete mice (**Figures 6A,B**). We did however find a significant difference in the levels of cortical insoluble Aβ_{40} ($t_{(19)} = 3.556$, $p < 0.01$, **Figure 6C**), where the 3xTg-AD complete

TABLE 2 | Body and brain weight differences between 3xTg-AD and NonTg mice.

	All subjects		
Genotype	**Body weight (g)**	**Brain weight (g)**	**Brain/Body weight ratio**
NonTg (*n* = 23)	27.14 ± 0.64	0.485 ± 0.003	0.018 ± 0.0004
3xTg-AD (*n* = 21)	30.12 ± 0.92	0.441 ± 0.004	0.015 ± 0.0004
p-value	0.0099**	<0.0001****	<0.0001****
Genotype		**IntelliCage Complete**	
NonTg (*n* = 18)	26.71 ± 0.76	0.485 ± 0.003	0.018 ± 0.0005
3xTg-AD (*n* = 12)	28.12 ± 0.91	0.442 ± 0.006	0.016 ± 0.0004
p- value	0.250	<0.0001****	0.0016**
Testing		**IntelliCage 3xTg-AD**	
Complete (*n* = 12)	28.12 ± 0.91	0.442 ± 0.006	0.016 ± 0.0004
Incomplete (*n* = 9)	32.79 ± 1.35	0.440 ± 0.004	0.014 ± 0.0006
p-value	0.008**	0.828	0.003**

*When including all subjects, 3xTg-AD mice show a significantly higher body weight and reduced brain weight compared to NonTg mice. The brain/body ratio is significantly lower for the 3xTg-AD mice. Two NonTg mice were excluded during place preference, bringing the n = 18. When including those that completed IntelliCage testing, 3xTg-AD mice show a significantly reduced brain and brain/body weight ratio than NonTg mice. When comparing 3xTg-AD mice that completed the IntelliCage testing versus those that did not (incomplete), we found a significantly higher body weight and a lower brain/body ratio for the incomplete. Data reported as Mean ± SEM. **p < 0.01, ****p < 0.0001.*

mice had a lower level than their incomplete counterparts. We also found a significant difference in the levels of cortical insoluble $A\beta_{42}$ ($t_{(19)} = 3.124$, $p < 0.01$, **Figure 6D**), where the 3xTg-AD complete mice had a higher level than the incomplete mice. Next, to determine whether tau phosphorylation may contribute to 3xTg-AD mice not being able to complete the IntelliCage tasks, we performed western blots (**Figure 6E**) for serine (Ser) and threonine (Thr) tau phosphorylation sites Ser202/Thr205 (AT8) and Thr212/Ser214 (AT100); AT8 and AT100 are detectable *via* western blot by 6 months of age in 3xTg-AD mice (Oh et al., 2010; Parachikova et al., 2010). We also verified the presence of human APP *via* immunoblot using these same protein homogenates (**Figure 6E**). Quantitative analysis revealed a significant elevation of AT8 in both the hippocampus ($t_{(44)} = 2.159$, $p < 0.05$; 6E, F) and cortex ($t_{(43)} = 2.057$, $p < 0.05$; **Figures 6E,F**) of 3xTg-AD mice compared to NonTg mice. For AT100, we found a significant elevation in the cortex of 3xTg-AD mice compared to NonTg mice ($t_{(42)} = 2.246$, $p < 0.05$; **Figures 6E,G**). Next, we analyzed AT8 and AT100 immunoblot expression between the 3xTg-AD complete vs incomplete samples and found no significant differences in either the hippocampus or cortex (**Figures 6E,H,I**). Lastly, we performed immunostaining for AT8 and found consistent results showing that AT8+ cells in the hippocampus were not statistically different between the 3xTg-AD complete versus incomplete mice (**Figures 6J,K**). Collectively, these results suggest that brain weight and fractions of cortical insoluble $A\beta_{40–42}$ may be associated with mice not being able to complete the adaptation phases in the IntelliCage.

DISCUSSION

Our results highlight, for the first time, that female 3xTg-AD mice show cognitive deficits in various tasks of the automated IntelliCage system. During the adaptation phases, only 57.14% of the 3xTg-AD mice were able to learn to drink water accessed through the noseport, while 86.96% of the NonTg mice drank and made it past all adaptation phases. Our data showed that the 3xTg-AD mice made fewer corner visits compared to NonTg mice during the first day of introduction into the IntelliCage. As testing progressed, 3xTg-AD mice consistently entered corners more frequently with the purpose to drink than the NonTg mice, which resulted in a higher percentage of correct corner visits during the reversal place preference phase. This suggests that during the place preference phases, the 3xTg-AD mice's sole purpose to enter a corner was to drink while NonTg mice entered corners to both obtain water and explore, thereby explaining this discrepancy in increased percent correct visits of 3xTg-AD mice in these phases of the IntelliCage. Previous reports show that increased body weight is associated with increased water consumption in transgenic mice (Bachmanov et al., 2002). While the body weight assessment at the end of our study showed the 3xTg-AD mice weighed significantly more than the NonTg mice, consistent with previous studies (Robison et al., 2020), 3xTg-AD mice that completed the adaptation phases were not significantly heavier than their NonTg counterparts. Additionally, water consumption between NonTg and 3xTg-AD mice was not significantly different during any of the IntelliCage tasks. Alternatively, the observed reduced exploratory behavior in 3xTg-AD mice could instead be an indicator of increased anxiety in 3xTg-AD mice (Giménez-Llort et al., 2013). Indeed, neophobia and anxiety can be measured immediately after introducing animals into a new environment, such as the IntelliCage (Kiryk et al., 2020). It is notable that 3xTg-AD mice only showed reduced exploratory behavior on day 1, as they likely acclimated to their new environment on subsequent days given equal corner visits compared to NonTg mice. These findings are consistent with previous reports showing that 3xTg-AD mice exhibit reduced exploration due to increased anxiety (Sterniczuk et al., 2010).

Executive functions such as attention and impulsivity, as well as working memory, are mediated by areas of the prefrontal cortex (Voikar et al., 2018). In AD, while the main focus has been on learning and memory deficits early in the progression of

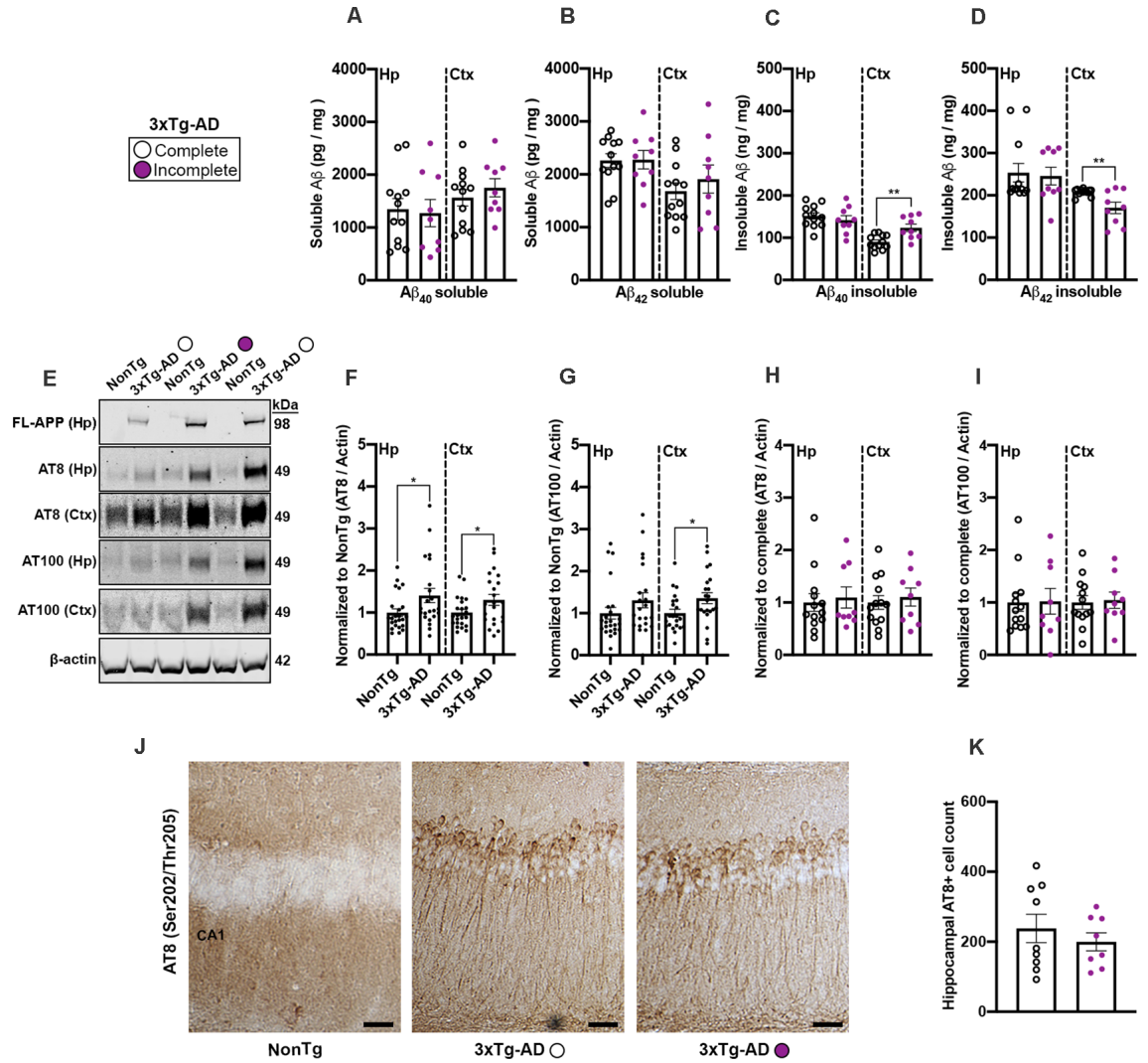

FIGURE 6 | 3xTg-AD mice that were unable to complete the IntelliCage adaptation phases showed increased insoluble Aß$_{40}$ levels and decreased insoluble Aß$_{42}$ levels. **(A,B)** No significant differences of soluble Aß$_{40–42}$ in the hippocampus (Hp) or cortex (Ctx) were found between 3xTg-AD that completed the IntelliCage tasks compared to 3xTg-AD incomplete mice. **(C)** We found that 3xTg-AD complete mice had a significantly lower level of insoluble Aß$_{40}$ in the cortex than the 3xTg-AD incomplete mice ($p < 0.01$). **(D)** We found that 3xTg-AD complete mice had a significantly higher level of insoluble Aß$_{42}$ in the cortex than the 3xTg-AD incomplete mice ($p < 0.01$). **(E)** Representative western blots of human APP, phospho(p) Ser202/Thr205 (AT8), Thr212/Ser214 (AT100), and ß-actin loading control. **(F)** 3xTg-AD mice showed higher expression of AT8 in the Hp and Ctx than NonTg mice. **(G)** 3xTg-AD mice show higher expression of AT100 in the Ctx than NonTg mice. **(H,I)** No significant difference was detected in AT8 or AT100 in the Hp and Ctx of 3xTg-AD complete compared to the 3xTg-AD incomplete mice**. (J)** Photomicrographs depicting the Hp Cornu Ammonis 1 (CA1) region stained for AT8+ cells. Scale bar = 50 μm. NonTg mice did not show detectable AT8+ cells in CA1 of the Hp. **(K)** No significant differences in AT8+ cell count were detected between 3xTg-AD complete compared to the 3xTg-AD incomplete mice. Data are presented as means ± SEM. *$p < 0.05$. **$p < 0.01$.

the disease, attentional dysfunction is also present as the disease evolves (Perry and Hodges, 1999; Berardi et al., 2005). The SRT and place avoidance tasks in the IntelliCage were used to assess these domains. The 3xTg-AD mice showed a lower percentage correct and a higher percent incorrect in the SRT task than the NonTg mice, which is consistent with previous reports showing attention deficits in this mouse model (Romberg et al., 2011). Notably, the number of total visits to all corners was significantly increased in 3xTg-AD particularly on day 2 and 3 of SRT testing; however, the number of trials initiated in the assigned corner were similar between the NonTg and 3xTg-AD mice on days 1–3. Given that the SRT task relies on mice to initiate a trial in only the assigned corner to gain water access, this suggests that the 3xTg-AD were likely entering other corners in search of water given that their percent correct was lower than the NonTg mice. Similarly, 3xTg-AD mice exhibited a higher number of working memory errors in the place avoidance task compared to their NonTg counterparts, also consistent with previous reports and illustrating deficits mediated by the prefrontal cortex (Stevens and Brown, 2015; Li et al., 2020).

Our data shows that 3xTg-AD mice had lower brain weights than their NonTg counterparts. This is consistent with a recent report showing that both male and female 3xTg-AD mice have lower brain mass than NonTg mice (Li et al., 2020). The findings that more 3xTg-AD mice were not able to successfully advance past the adaptation phases and had reduced brain weights are consistent with a report illustrating that brain mass may be an indicator of cognitive capacities (Perepelkina et al., 2020). Future work will focus on whether such brain weight differences in 3xTg-AD are the result of atrophy, or if there may be developmental issues in this transgenic mouse model contributing to such discrepancies. Further neuropathological analysis of AD-like pathology markers revealed that 3xTg-AD mice that completed the IntelliCage tasks had a lower level of cortical insoluble $A\beta_{40}$ and a higher level of insoluble $A\beta_{42}$. A previous report found that female 3xTg-AD mice's insoluble $A\beta_{40}$ and $A\beta_{42}$ levels increased significantly with age from 6–12 months (Oddo et al., 2003). However, insoluble $A\beta_{40}$ levels have been shown to increase early and play a mechanistic role in the progression of AD (Wang et al., 1999). Given that 3xTg-AD mice in our study were between 7–9 months of age when IntelliCage testing commenced suggests that insoluble $A\beta_{40}$ levels may be an early predictor of performance in these mice and that $A\beta_{42}$ levels may become more relevant at later ages. When examining phosphorylation markers of tau, we found significant elevations of phosphorylation at Ser202/Thr205 (AT8) in the hippocampus and cortex of 3xTg-AD mice compared to NonTg that completed the IntelliCage tasks. For phosphorylated tau at Thr212/Ser214 (AT100), we only found significant differences between NonTg and 3xTg-AD in the cortex. This is not surprising, as tau phosphorylation at AT8 is widespread in the hippocampus by 6 months of age, while tau phosphorylated at AT100 is more widespread in the hippocampus by 12 months (Oh et al., 2010; Parachikova et al., 2010). These results suggest that phosphorylated tau may play a role in differences observed between NonTg and 3xTg-AD mice. However, we did not find any significant phosphorylated tau differences in 3xTg-AD mice that completed the IntelliCage tasks compared to those that failed to pass the adaptation phases. Given the age of the 3xTg-AD mice in the current study and the limited amount of tau pathology at this age, it is not likely that tau was a driving factor for the 3xTg-AD that completed versus those that did not complete the tasks.

In summary, this work is the first to report behavioral deficits in 3xTg-AD mice on various tasks of the IntelliCage. We found that 3xTg-AD mice explore less on the first day in the novel environment, indicating increased anxiety, in addition to deficits in attention and working memory in later tasks. More than one-third of the 3xTg-AD mice did not complete the IntelliCage tasks and showed differences in brain weight, brain-to-body weight ratios, and insoluble $A\beta_{40-42}$. Collectively, these results fill in the gap of a much-needed area of research, testing a widely used mouse model of AD in the IntelliCage, and will inform scientists of important factors to consider when testing the 3xTg-AD mouse in this automated behavioral phenotyping system.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by IACUC.

AUTHOR CONTRIBUTIONS

WW: experimental design, IntelliCage testing, ELISA, figure creation, writing and editing the manuscript. IM: IntelliCage testing, algorithm development and data extraction, writing and editing the manuscript. ST: figure creation, animal harvesting, immunohistochemistry, writing and editing the manuscript. AD: IntelliCage testing assistance, animal harvesting assistance, westernblotting, immunohistochemistry, and editing the manuscript. AV: animal harvesting assistance, image quantification, statistical analysis, figure creation, and editing the manuscript. RV: experimental design, funding, IntelliCage testing, statistical analysis, figure creation, writing and editing the manuscript. All authors contributed to the article and approved the submitted version.

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Oxidative Phosphorylation Is Dysregulated Within the Basocortical Circuit in a 6-month old Mouse Model of Down Syndrome and Alzheimer's Disease

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Down syndrome (DS) is the primary genetic cause of intellectual disability (ID), which is due to the triplication of human chromosome 21 (HSA21). In addition to ID, HSA21 trisomy results in a number of neurological and physiological pathologies in individuals with DS, including progressive cognitive dysfunction and learning and memory deficits which worsen with age. Further exacerbating neurological dysfunction associated with DS is the concomitant basal forebrain cholinergic neuron (BFCN) degeneration and onset of Alzheimer's disease (AD) pathology in early mid-life. Recent single population RNA sequencing (RNA-seq) analysis in the Ts65Dn mouse model of DS, specifically the medial septal cholinergic neurons of the basal forebrain (BF), revealed the mitochondrial oxidative phosphorylation pathway was significantly impacted, with a large subset of genes within this pathway being downregulated. We further queried oxidative phosphorylation pathway dysregulation in Ts65Dn mice by examining genes and encoded proteins within brain regions comprising the basocortical system at the start of BFCN degeneration (6 months of age). In select Ts65Dn mice we demonstrate significant deficits in gene and/or encoded protein levels of Complex I-V of the mitochondrial oxidative phosphorylation pathway in the BF. In the frontal cortex (Fr Ctx) these complexes had concomitant alterations in select gene expression but not of the proteins queried from Complex I-V, suggesting that defects at this time point in the BF are more severe and occur prior to cortical dysfunction within the basocortical circuit. We propose dysregulation within mitochondrial oxidative phosphorylation complexes is an early marker of cognitive decline onset and specifically linked to BFCN degeneration that may propagate pathology throughout cortical memory and executive function circuits in DS and AD.

Keywords: oxidative phosphorylation, basal forebrain, Down syndrome, Alzheimer's disease, selective vulnerability

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INTRODUCTION

Down syndrome (DS) is caused by the triplication of human chromosome 21 (HSA21) and is the primary genetic cause of intellectual disability (ID). HSA21 triplication is present in approximately 1 in 700 live births and these individuals exhibit multiple systemic functional deficits, including heart conditions, increased incidence of leukemias, epilepsy, premature aging, and neurological deficits (Bittles et al., 2007; So et al., 2007; Lott, 2012; Presson et al., 2013; Mai et al., 2019). While the lifespan of individuals with DS has increased significantly in the past several decades, healthspan lags appreciably behind (Hill et al., 2003; Bittles et al., 2007; Presson et al., 2013; Dick et al., 2016). Most adults with DS start to exhibit Alzheimer's disease (AD)-like pathology, including senile plaques, neurofibrillary tangles, synaptic dysfunction, and basal forebrain cholinergic neuron (BFCN) degeneration, with the accompanying cognitive decline, typically by the mid-third decade of life (Mann et al., 1984; Coyle et al., 1988; Beacher et al., 2009; Lott and Dierssen, 2010; Costa, 2012; Lott, 2012; Hartley et al., 2015; Annus et al., 2016). BFCN degeneration is an early pathological feature of both DS and AD and coincides with cognitive decline early in disease onset and throughout progression (Yates et al., 1980; Sendera et al., 2000; Mufson et al., 2002, 2008; Iulita et al., 2014). Recent imaging evidence indicates volume reductions in the basal forebrain (BF) predicts entorhinal cortex loss and cortical spread of degeneration in AD and correlates with AD biomarkers (Grothe et al., 2013; Cavedo et al., 2020; Fernández-Cabello et al., 2020; Teipel et al., 2020), suggesting BF dysfunction is one of the earliest pathological changes during the development of AD.

Mitochondrial oxidative phosphorylation is the main energy source within neurons and is critical for normal brain development and function (Mattson et al., 2008; Hall et al., 2012). Deficits within the oxidative phosphorylation pathway have devastating effects on normal neuronal function (Mattson et al., 2008). The oxidative phosphorylation apparatus has five mitochondrial respiratory chain complexes (Complexes I-V), comprised of NADH-ubiquinone oxidoreductase Complex I, succinate dehydrogenase Complex II, cytochrome bc_L Complex III, cytochrome C oxidase Complex IV, and the ATP synthase Complex V (Valenti et al., 2014). Individual mitochondrial complex proteins within the oxidative phosphorylation pathway have been shown to be dysregulated in DS cell culture models (Valenti et al., 2016; Briggs et al., 2017). Little information is currently available on the status of individual oxidative phosphorylation markers in the context of DS *in vivo* (Kim et al., 2000, 2001; Bambrick and Fiskum, 2008). Although little doubt exists that oxidative phosphorylation is critical for normal brain function, oxidative phosphorylation pathway deficits in the DS brain demands further exploration. Indeed, recent evidence suggests evaluating oxidative phosphorylation complex proteins in the cortex of the well-established Ts65Dn mouse model of DS and AD is difficult and variable (Lanzillotta et al., 2021), further highlighting the necessity of additional interrogation of the oxidative phosphorylation pathway in the brain of DS and AD relevant model systems.

There are several mouse models of DS available, however, the Ts65Dn mouse is the most prevalent in terms of use and applicability (Rueda et al., 2012; Ruparelia et al., 2012; Ahmed et al., 2017). The Ts65Dn mouse model recapitulates many of the endophenotypes of DS and AD, including hippocampal-dependent learning and memory deficits, BFCN degeneration and septohippocampal circuit dysfunction, notably CA1 pyramidal neuron and choline acetyltransferase (ChAT) activity deficits (Granholm et al., 2000; Belichenko et al., 2004, 2009; Kelley et al., 2014a,b). Degeneration of the BFCN system, including within the septohippocampal and basocortical pathways, are hallmarks of disease progression in DS and AD and are a primary feature of the Ts65Dn mouse model (Holtzman et al., 1996; Granholm et al., 2000; Hunter et al., 2003a; Strupp et al., 2016). BFCN degeneration begins at approximately 6 months of age (MO) in Ts65Dn mice, and loss of BFCNs and deficits in hippocampal cholinergic innervation are uniformly reported by ∼10 MO (Holtzman et al., 1996; Cooper et al., 2001; Hunter et al., 2003b; Contestabile et al., 2006; Powers et al., 2016). Many of the mitochondrial deficits reported in human DS, principally in the periphery (Izzo et al., 2018; Bayona-Bafaluy et al., 2021) are also present in this trisomic model including lower ATP production *in vitro* (Valenti et al., 2016) and metabolic changes in peripheral cell types (Cisterna et al., 2020). Further, genes postulated to be involved in mitochondrial dysfunction, including *Dyrk1a* and *Ets2* (Izzo et al., 2018) are triplicated in human DS and reproduced in the Ts65Dn mouse model. These two genes have recently been shown to be upregulated within BFCNs microisolated from the medial septal nucleus (MSN; Alldred et al., 2021).

Although the oxidative phosphorylation pathway is known to be dysregulated in DS (Helguera et al., 2013; Izzo et al., 2018; Bayona-Bafaluy et al., 2021) and changes in oxidative phosphorylation states have significant deleterious effects on normal neuronal function (Mattson et al., 2008), prior studies lack pathway-based evaluations conducted *in vivo* to understand mechanisms driving this critical pathology. Herein, we utilized the Ts65Dn mouse model to examine connectivity based degeneration deficits in two interconnected brain regions comprising the basocortical system, the BF and Fr Ctx, which are critically impacted in DS and AD and are reflective of age-related cognitive decline and loss of executive function. We examined BF and Fr Ctx levels for oxidative phosphorylation changes at the transcript and encoded protein levels within each oxidative phosphorylation complex. We postulate reductions observed within the basocortical circuit indicate BF degeneration drives oxidative phosphorylation dysregulation and paces or precedes cortical dysfunction associated with DS and AD.

MATERIALS AND METHODS

Mice

Animal protocols were approved by the Nathan Kline Institute/NYU Grossman School of Medicine IACUC in accordance with NIH guidelines. Breeder pairs (female Ts65Dn and male C57Bl/6J Eicher x C3H/HeSnJ F1 mice) were purchased

from Jackson Laboratories (Bar Harbor, ME, USA) and mated at the Nathan Kline Institute. Mice were given *ad libitum* food and water access (Alldred et al., 2015a,b). Standard cages contained paper bedding and several objects for enrichment (e.g., plastic igloo, t-tube, and cotton square). Mice were maintained on a 12-h light-dark cycle under temperature- and humidity-controlled conditions. Tail clips were taken and pups were genotyped (Duchon et al., 2011) at weaning (P21) and aged to ∼6 MO.

Tissue Preparation

At ∼6 MO, mice were sacrificed for brain tissue accession. Mice were given an overdose of ketamine and xylazine and perfused transcardially with ice-cold 0.15 M phosphate buffer (Alldred et al., 2015a,b, 2018, 2019). Brain tissues were accessed from Ts65Dn (Ts; n = 10) and age-matched normal disomic (2N; n = 10) male mice, with littermates between 2N and Ts mice used when possible (age range: 5.8–6.4 MO, mean age 6.0 MO). The BF was dissected to enrich for cholinergic neurons in the medial septal/ventral diagonal band region (∼Bregma 1.35–0.26) as well as the left Fr Ctx dissected using standard coordinates from the mouse brain atlas (Paxinos and Franklin, 2001). Dissections were either flash-frozen or kept on wet ice for homogenization directly following brain accrual. Tissue was homogenized using ice-cold Tris homogenization buffer [THB; 20 mM Tris-Cl (pH 7.4), 1 mM EGTA, 1 mM EDTA and 0.25 M sucrose] with a protease inhibitor cocktail (1:1,000, I3786, Sigma-Aldrich, St. Louis, MO, USA and 1 mM PMSF, ThermoFisher, Waltham, MA, USA) using 1.5 mm zirconium beads on Beadbug homogenizer (Benchmark Scientific, Sayreville, NJ, USA) for 30 s at 4,000 rpm. Post homogenization, samples were kept on ice and cell debris was spun down at 2,500× g for 5 min at 4°C. The supernatant was aliquoted to fresh tubes for isolation of RNA (done immediately following homogenization) or protein assays (each assay done with fresh aliquots of tissue homogenates stored at −80°C). RNase-free precautions were employed, and solutions were made with 18.2 mega Ohm RNase-free water (Nanopure Diamond, Barnstead, Dubuque, IA, USA).

RNA Purification

RNA from microdissected regions of mouse tissue from BF and Fr Ctx were purified using the miRNeasy Mini kit (Qiagen, Hilden, Germany) according to manufacturers' specifications. A DNase digestion was performed twice sequentially before the final washes and RNA purification (Alldred et al., 2021). RNA quality control was performed at a 1:5 dilution to preserve RNA for downstream applications (RNA 6000 pico kit, Agilent, Santa Clara, CA, USA).

RT-qPCR

Equal amounts of RNA was reverse transcribed in a 50 μl reaction volume to generate cDNA from Fr Ctx and BF tissue from Ts and 2N littermates (n = 10 per genotype per brain region) using random hexamers as described previously (Alldred et al., 2012, 2015a,b, 2018, 2019; Bordi et al., 2016). RT-qPCR was performed using 1 μl of cDNA and Taqman PCR primers for select genes from oxidative phosphorylation Complexes I-V along with a mitochondrial rRNA gene (**Table 1**) to assay samples in triplicate on a real-time qPCR cycler (PikoReal, ThermoFisher) as previously described (Alldred et al., 2008, 2012, 2015a,b, 2018, 2019; Jiang et al., 2010). The ddCT method was used to determine relative gene level differences between genotypes (ABI, 2004; Ginsberg et al., 2010; Jiang et al., 2010). Glucuronidase beta (*Gusb*, Mm01197678_m1*)* and 45S pre-ribosomal RNA *(Rn45s*; **Table 1**) qPCR products were interrogated for use as controls as they did not show significant changes by genotype within MSN BFCN RNA-seq data (Alldred et al., 2021). *Rn45s* was subsequently selected as the control housekeeping gene. Negative controls consisted of the reaction mixture without input RNA. For each gene, the PCR product synthesis was modeled as a function of genotype, using mixed effects models with random mouse effect to account for the correlation between repeated assays on the same mouse (McCulloch et al., 2011; Alldred et al., 2015a,b, 2018, 2019). Significance was judged at the level α=0.05, two-sided.

Protein Assays

Protein expression analysis was performed using the WES system (Protein Simple, Santa Clara, CA, USA; Nguyen et al., 2011). Briefly, protein samples were diluted in THB buffer 1:100 (w/v), with 1× final concentration of fluorescent molecular weight marker (provided in the kit) and heated to 50°C for 5 min (as per manufacturers' recommendation), then cooled to 4°C before loading onto the WES system plate with a molecular weight ladder. All blocking reagents, chemiluminescent substrate, separation, and stacking matrices (Protein Simple) were dispensed to designated wells. Primary antibodies against Complex I-V (Total OXPHOS panel, 1:20 dilution, ab110413, AbCam, Cambridge, United Kingdom) containing five mouse mAbs, Complex I (NADH:ubiquinone oxidoreductase subunit B8; NDUFB8), Complex II (succinate dehydrogenase beta; SDHB), Complex III (Cytochrome b-c1 complex subunit 2;UQCR2), Complex IV (Mitochondrially encoded cytochrome C oxidase I; MTCO1) and Complex V (ATP synthase lipid-binding protein; ATP5A), along with a control antibody against β-Tubulin III (β-TubIII; R&D Systems, Minneapolis, MN, USA, MAB1195 1:50) and HRP conjugated secondary antibody (rabbit anti-mouse; DM-002; Protein Simple) were dispensed to designated wells. Plates were spun for 5 min at 1,000× g and loaded onto the WES unit, where separation electrophoresis and immunodetection steps are fully automated within the capillary system. Instrument default settings were used with the exception of protein loading run time that was increased from 25 to 35 min. The digital image was analyzed with Compass software (Protein Simple), utilizing dropped lines for peak analysis area calculation. Detected proteins were compared to control protein (β-TubIII) and reported as the normalized percentage of control. Each protein was performed in triplicate on separate plate runs. Statistical analysis was conducted on each protein normalized to β-TubIII and modeled as a function of the mouse study group (Ts and 2N, n = 10 per genotype per brain region), using mixed effects models with random mouse effect to account for the correlation between repeated assays on the same mouse

TABLE 1 | List of TaqMan primers for RT-qPCR analysis.

Gene	TaqMan Primer	Description
Rn45S	Rn03928990_g1	Housekeeping, 45S pre-ribosomal RNA
Mt-Nd1	Mm04225274_s1	Complex I, mitochondrial NADH dehydrogenase 1
Mt-Nd4l	Mm04225294_s1	Complex I, mitochondrial NADH 4L dehydrogenase
Sdha	Mm01352360_m1	Complex II, succinate dehydrogenase complex flavoprotein, subunit A
Mt-Cytβ	Mm04225271_g1	Complex III, mitochondrial cytochrome b
Mt-Cox2	Mm03294838_g1	Complex IV, mitochondrial Cytochrome c oxidase subunit II
Mt-Atp8	Mm04225236_g1	Complex V, mitochondrial ATP synthase 8
Mt-Rnr1	Mm04260177_s1	Mitochondrial 12S rRNA

(McCulloch et al., 2011; Alldred et al., 2015a,b, 2018, 2019). Significance was judged at the level $\alpha = 0.05$, two-sided.

Deproteinization and ATP Assay

Biochemical analysis of ATP levels were conducted using Fr Ctx tissue. Deproteinization of mouse Fr Ctx homogenates were performed utilizing the Deproteinizing Sample preparation kit (ab204708, AbCam) according to manufacturer's specifications with the following alterations, starting sample volume was reduced to 50 μl from 100 μl with 7.5 μl of TCA, and neutralization was performed with 5 μl of neutralization solution. Immediately following deproteinization, an ATP assay (ATP assay kit, ab83355, AbCam) was performed in duplicate for each sample ($n = 6$ per genotype) utilizing a 1:4 dilution of the deproteinized sample according to the manufacturer's specifications for the fluorometric assay. The fluorometric samples were read in duplicate on a plate reader (SpectraMax, Molecular Devices, San Jose, CA, USA). ATP concentration was calculated according to the manufacturer's specifications. Statistical analysis was performed using a non-parametric method (Wilcoxon Test) due to the small sample size and some losses of the sample (Wilcoxon, 1946; Pratt, 1959). Significance was judged at the level $\alpha = 0.05$, two-sided.

RESULTS

To understand changes in oxidative phosphorylation within the basocortical circuit, we examined RNA and protein levels from each of the five complexes in the oxidative phosphorylation pathway from two synaptically connected brain regions critical for attention, memory, and executive function that show a significant decline in DS and AD. This examination was based upon gene expression profile changes in the oxidative phosphorylation pathway from MSN BFCNs by single population RNA-seq in ∼6 MO Ts65Dn mice compared to 2N littermates (Alldred et al., 2021). Bioinformatic inquiry by Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of the oxidative phosphorylation pathway revealed downregulation of multiple subunits of Complex I, III, IV, and V, but no significant changes in Complex II subunits within MSN BFCNs (**Figure 1**).

To examine if transcriptomic changes seen in the Ts65Dn MSN BFCNs are selective to the MSN enriched BF or are seen throughout the basocortical circuit, we examined both BF and Fr Ctx at the RNA and protein level for expression changes at the start of BFCN degeneration. We examined a total of six subunits from Complexes I-V within the oxidative phosphorylation pathway for gene expression, utilizing transcripts that were significantly downregulated in MSN BFCNs *via* single population RNA-seq along with candidates that were not differentially regulated, to examine the pathway in a comprehensive manner. We also assessed the *Mt-Rnr1* gene, which encodes the 12S rRNA as a marker of total mitochondrial RNA. In the BF, downregulation was observed for members of Complex I, *Mt-Nd1* (trend level $p = 0.072$) and *Mt-Nd4l* ($p < 0.00228$), Complex II, *Sdha* ($p < 0.00846$), Complex III, *Mt-Cytβ* (not significant), Complex IV, *Mt-Cox2* (not significant), and Complex V, *Mt-Atp8* ($p < 0.0268$; **Figure 2A**). Total mitochondrial rRNA (*Mt-Rnr1*) also showed a downregulated trend ($p = 0.064$; **Figure 2A**). When correlating the single population MSN BFCN RNA-seq to regional BF RT-qPCR log fold changes (LFC), a moderately high correlation was observed ($R^2 = 0.5759$; **Figure 2B**). Interestingly, while not significant, downregulation observed *via* RT-qPCR for *Mt-Nd1* and *Mt-Cox2* correlated with the significant downregulation seen in trisomic MSN BFCNs by RNA-seq. The regional BF gene expression of *Mt-Nd1* was only trend level downregulated *via* RT-qPCR, suggesting these changes are neuron-specific and the observed downregulation within MSN BFCNs is diluted when examined in tissue with admixed neuronal and non-neuronal cell types. The same subset of genes was interrogated in the Fr Ctx of Ts65Dn mice compared to 2N littermates by RT-qPCR. Of the six genes examined from the five complexes, no changes were found in either Complex I or II genes (*Mt-Nd1*, *Mt-Nd4l*, and *Sdha*), trend level decreases in Complex III and IV genes (*Mt-Cytβ* $p = 0.067$ and *Mt-Cox2* $p = 0.079$), and significant downregulation of Complex V member *Mt-Atp8* ($p < 0.0059$; **Figure 2C**). Total mitochondrial rRNA was significantly downregulated in the trisomic Fr Ctx ($p < 0.0012$; **Figure 2C**). Similar to regional BF RT-qPCR, a moderately high correlation between MSN BFCN RNA-seq and Fr Ctx RT-qPCR LFC was found ($R^2 = 0.6679$; **Figure 2D**).

We employed the Total OXPHOS panel (Abcam) to assess protein expression of select subunits from Complexes I-V in the BF and Fr Ctx within Ts65Dn mice and 2N littermates. When examining the digital traces in BF homogenates (**Figure 3A**), we saw a relatively high expression of Complex II-V proteins, but relatively low expression of Complex I protein (NDUFB8), indicating this may not be a major subunit expressed in basocortical brain tissue. Within

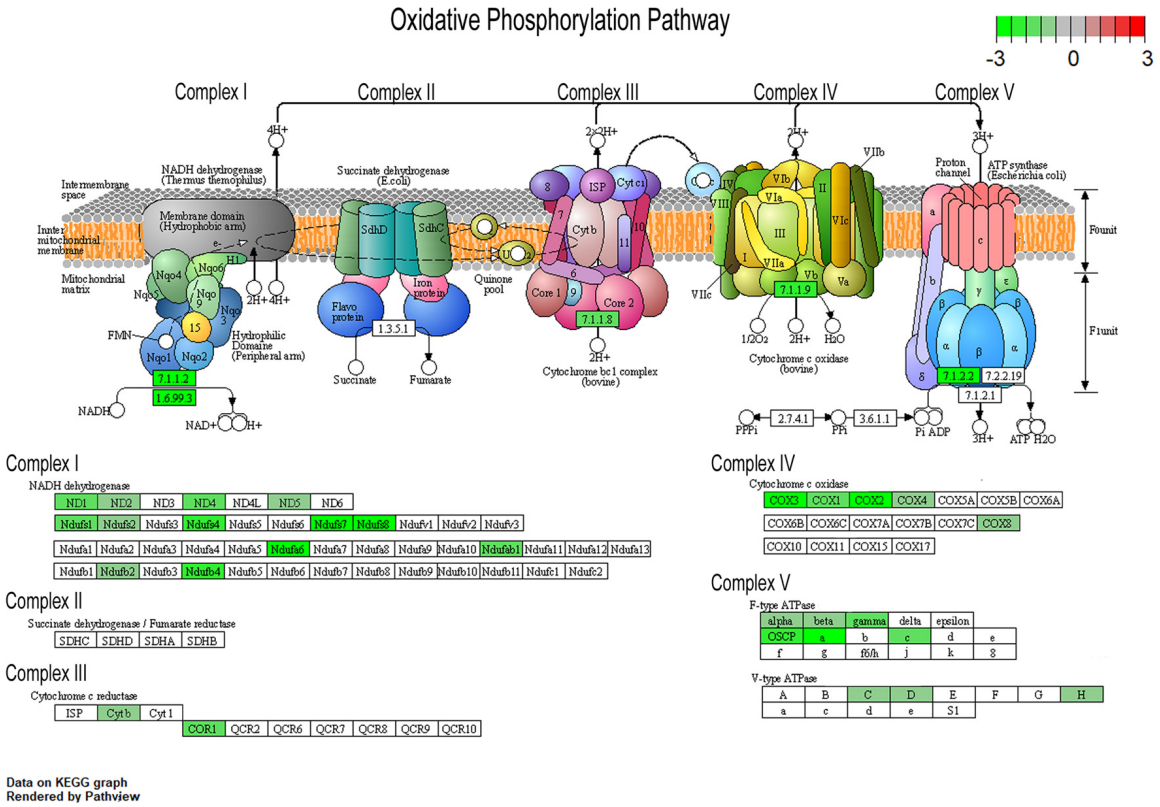

FIGURE 1 | Pronounced downregulation of members of the oxidative phosphorylation pathway in trisomic basal forebrain cholinergic neurons (BFCNs). The Kyoto Encyclopedia of Genes and Genomes (KEGG) derived oxidative phosphorylation pathway is rendered by Pathview, with significantly dysregulated genes highlighted for each complex from the medial septal nucleus (MSN) BFCNs as assayed by single population RNA-seq (Alldred et al., 2021). Downregulation is shown as color coded log fold change difference (LFC) with green depicting downregulation and red depicting upregulation. Note no genes within the oxidative phosphorylation pathway were upregulated.

the BF, Complex I, NDUFB8 expression was not significantly downregulated. However, downregulation of proteins from Complex II (SDHB, $p < 0.016$), III (UQCRC2, $p < 0.0055$), IV (MT-CO1, $p < 0.0034$), and V (ATP5A, $p < 0.019$) were found (**Figure 3B**), which highly correlated with MSN BFCN RNA-seq data ($R^2 = 0.7658$; **Figure 3C**). When examining the same Complex I-V proteins in Fr Ctx tissue, variability in relative expression levels was observed between animals (**Figure 3D**) and no significant downregulation of protein expression was detected. The only significant change in protein expression in trisomic Fr Ctx tissue homogenates was upregulation of Complex I (NDUFB8, $p < 0.0012$; **Figure 3E**). In contrast to RT-qPCR findings, protein levels in Fr Ctx did not correlate with MSN BFCN RNA-seq data ($R^2 = 0.0422$, **Figure 3F**).

We indirectly examined the functionality of the oxidative phosphorylation complex by biochemical analysis of ATP levels, which are the output of the ATP synthase Complex V. Due to the small size of mouse BF and the quantity of tissue needed for the ATP assay, we were only able to perform this measurement in Fr Ctx tissue. We found a significant decrease in ATP levels in trisomic Fr Ctx ($p < 0.0307$; **Figure 3G**), which matched Fr Ctx RT-qPCR findings for Complex V.

DISCUSSION

We employed the Ts65Dn mouse model of DS and AD to examine oxidative phosphorylation pathway changes within the basocortical circuit. The BF provides the main cholinergic inputs for the hippocampus and cerebral cortex (Mesulam et al., 1983). Cholinergic fiber input into the cortex is involved in both attentional behavior and cognition and the activity of these cholinergic neurons is decreased during normal human aging (Mufson et al., 2003). This deficit is exacerbated during AD and DS progression (Coyle et al., 1986, 1988; Mufson et al., 2003). We examined expression level differences of individual mitochondrial oxidative phosphorylation subunits at the transcript and encoded protein levels ∼6 MO, a timepoint where BFCN degeneration is initiated in Ts65Dn mice. Bioinformatic inquiry of our recent single population RNA-seq analysis of MSN BFCNs in Ts65Dn and 2N littermates indicates that oxidative phosphorylation and mitochondrial dysfunction are two of the top canonical pathways by Ingenuity Pathway Analysis (IPA) downregulated in this vulnerable cell type directly relevant to human DS and AD pathophysiology (Alldred et al., 2021). Understanding the age of degeneration

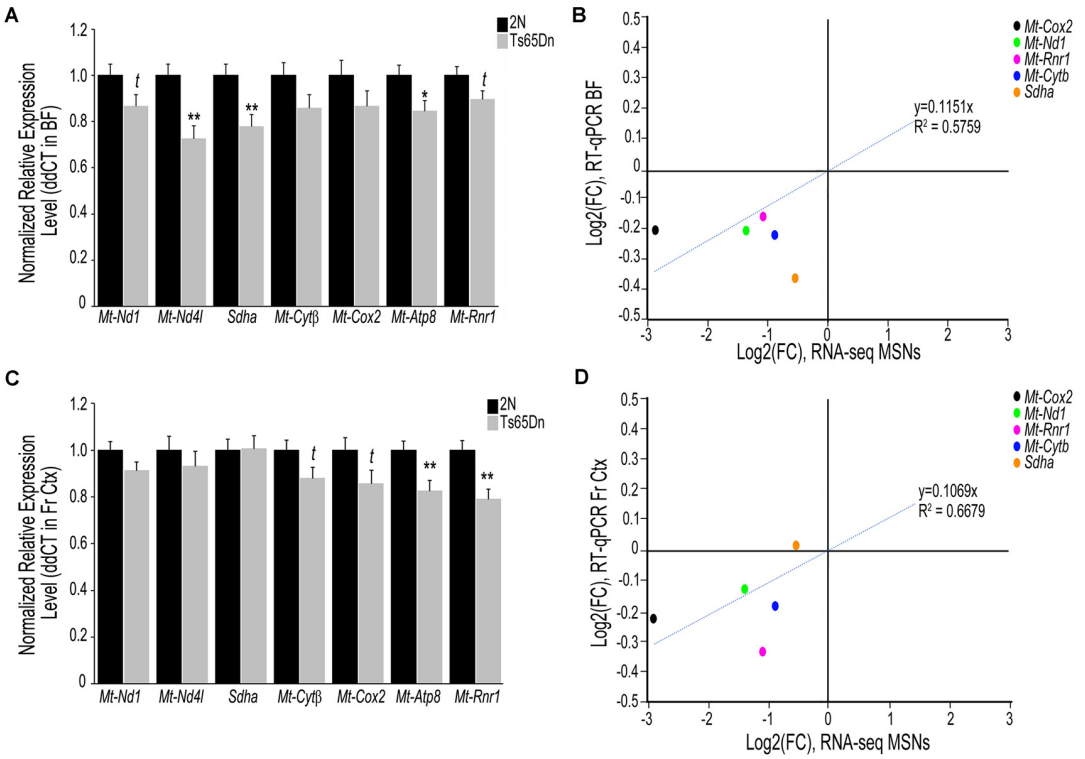

FIGURE 2 | Interrogation of oxidative phosphorylation Complex I-V subunit gene expression within the trisomic basocortical circuit. RT-qPCR was performed to determine gene expression levels using regional dissections of the basal forebrain (BF; **A,B**) and Fr Ctx **(C,D)** from Ts65Dn and 2N littermates at ∼6 MO for six genes. **(A)** Bar graph represents ddCT of each gene normalized to 2N levels in the BF. Significant downregulation was found for *Mt-Nd4l, Sdha*, and *Mt-Atp8* and trend-level downregulation was found for *Mt-Nd1*, *Mt-Cyt*β, and *Mt-Rnr1*. **(B)** Correlation plot association between MSN BFCN RNA-seq LFC (x-axis) and BF RT-qPCR LFC (y-axis). **(C)** Bar graph represents ddCT of each gene normalized to 2N levels in the Fr Ctx. Significant downregulation was found for *Mt-Atp8* and *Mt-Rnr1* and trend-level downregulation was found for *Mt-Cyt*β and *Mt-Cox2*. **(D)** Correlation plot association between MSN BFCN RNA-seq LFC (x-axis) and Fr Ctx RT-qPCR LFC (y-axis). While most genes were not significantly downregulated by RT-qPCR, they trended in the same direction (downregulation). *Sdha* was the only gene that did not correlate with RNA-seq results. Black bars represent relative 2N expression and gray bars indicate Ts65Dn expression normalized to 2N for each gene (standard error of mean (SEM) is indicated by error bars). Key: $^{*}p < 0.05$, $^{**}p < 0.01$; *t*, trend.

onset and the pathways involved at this inception point would help pinpoint novel targets for therapeutic development for slowing or stopping the degeneration associated with cognitive decline and loss of executive function. Indeed, despite hundreds of trials for AD and DS treatment, no new therapeutics have proven effective for AD or DS, with researchers suggesting that the delayed onset of treatment is a driving factor of the failure of these clinical trials (Gauthier et al., 2016; Yiannopoulou et al., 2019).

Oxidative stress and mitochondrial dysfunction are thought to play a critical role in DS and AD pathology (Mattson et al., 2008; Lott, 2012; Helguera et al., 2013; Izzo et al., 2018). Interestingly, a recent review postulates DS is an oxidative phosphorylation disorder (Bayona-Bafaluy et al., 2021). To date, the majority of studies evaluating oxidative phosphorylation and mitochondrial dysfunction in DS have used *in vitro* model systems. Although helpful to ascertain mechanistic interactions, these *in vitro* studies benefit from parallel *in vivo* assessments using the animal model and postmortem human brain tissue in the context of DS and AD for greater applicability and disease relevance.

Based on the present results, we postulate downregulation of select members of the oxidative phosphorylation pathway in the BF precedes degenerative changes within Fr Ctx, in a connectivity based degeneration course of action. Supporting evidence comes from the observation that downregulation of oxidative phosphorylation pathway complex genes is less pervasive in Fr Ctx along with a generalized lack of encoded protein changes at ∼6 MO, whereas the BF has profound transcript and protein level changes (**Figures 2A,C**, **3B,E**). These results correlate strongly with our previous RNA-seq analysis (**Figures 2B**, **3B**) within MSN BFCNs, indicating mitochondrial oxidative phosphorylation complexes are highly vulnerable in DS. Complementary observations were found in the Fr Ctx by an independent group using the Ts65Dn model at different age timepoints including no changes in Complex II (SDHB) or Complex V (ATP5A) subunits and with deficits in Complex III (UQCRC2) and Complex IV (MT-COX2) not seen until 18 MO (Lanzillotta et al., 2021). Our ∼6 MO data is commensurate with their 9 MO data showing a significant increase in Complex I (NDUFB8) levels, which is reversed at 18 MO, possibly indicating data this independent research group postulates is

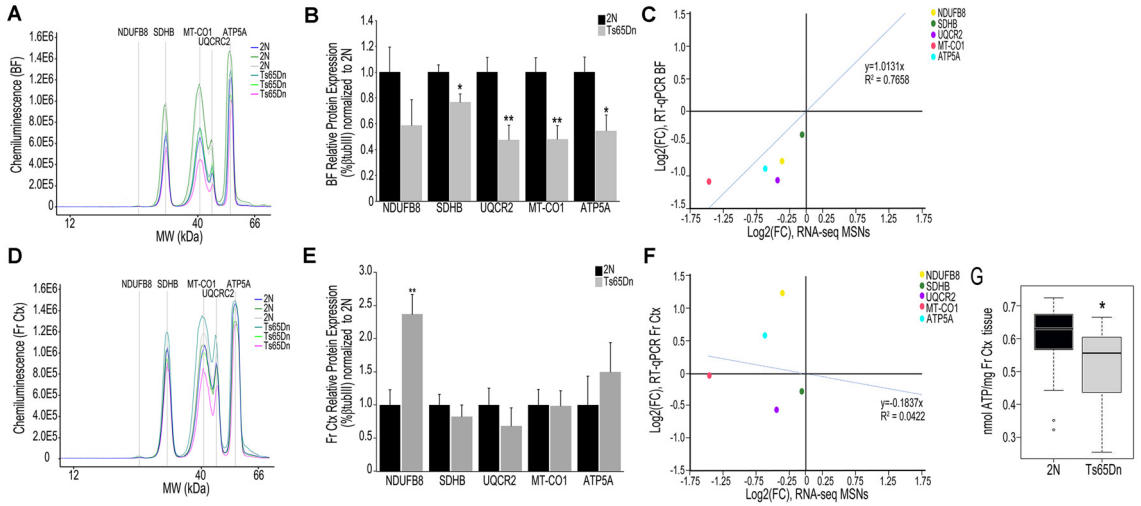

FIGURE 3 | Interrogation of oxidative phosphorylation Complex I-V subunit protein expression within the trisomic basocortical circuit. **(A)** Representative digital signatures of each assayed protein raw expression levels (y-axis) and molecular weight (x-axis) from Ts65Dn and 2N BF tissue homogenates. **(B)** Bar graph represents relative protein levels (normalized to beta-tubulin (βTubIII) as a percentage of 2N expression in BF tissue homogenates. Black bars represent 2N and gray bars indicate Ts65Dn (SEM indicated by error bars). **(C)** Correlation plot association between MSN BFCN RNA-seq LFC (x-axis) and BF protein level LFC (y-axis) indicating a high correlation. **(D)** Representative digital signatures of each assayed protein raw expression levels (y-axis) and molecular weight (x-axis) from Ts65Dn and 2N Fr Ctx tissue homogenates. **(E)** Bar graph represents relative protein levels (normalized to βTubIII) as a percentage of 2N expression in Fr Ctx tissue homogenates (SEM indicated by error bars). **(F)** Correlation plot association between MSN BFCN RNA-seq LFC (x-axis) and Fr Ctx protein level LFC (y-axis) indicating no significant correlation. **(G)** Box and whisker plots highlight downregulation of ATP levels in trisomic Fr Ctx. Key: $^{*}p < 0.05$, $^{**}p < 0.01$.

due to the shift from juvenile age to adulthood and to aged animals, whereby Ts65Dn mice react differently than their 2N counterparts (Lanzillotta et al., 2021). These researchers also conclude the oxidative phosphorylation machinery is highly downregulated in their DS cohort (Lanzillotta et al., 2021). BF was not evaluated, so comparisons are only available in the Fr Ctx. Although a compensatory mechanism prior to overt pathology onset is possible and would explain upregulation of NDUFB8 protein levels in Fr Ctx, differential regulation of this subunit does not reflect overall deficits in the oxidative phosphorylation pathway seen in our ∼6 MO trisomic cohort during initiation of BFCN degeneration. We also demonstrate downregulation of cholinergic and glutamatergic protein levels in the BF (**Supplementary Figure 1**), indicating synaptic deficits also exist in ∼6 MO trisomic mice, correlating with single population RNA-seq findings (Alldred et al., 2021). Synaptic-related marker downregulation in the BF which project to the Fr Ctx, along with the high number of significantly downregulated genes and proteins evidenced in BF, and the reduction of corresponding deficits in Fr Ctx are supportive of our overarching hypothesis that BF degeneration drives oxidative phosphorylation dysregulation and precedes cortical dysfunction in DS as well as across the AD spectrum (Mufson et al., 2016, 2019). Deficits in the activity of the oxidative phosphorylation complex have been shown in astrocyte cultures derived from the Ts1Cje DS mouse model, including lower ATP levels and decreased mitochondrial membrane potential (Shukkur et al., 2006). This study also showed *in vivo* brain ATP levels are reduced in Ts1Cje mice at 3 MO (Shukkur et al., 2006). Neural progenitor cells obtained from the Ts65Dn hippocampus revealed reduced ATP levels and loss of mtDNA levels, indicating a neuronal deficit in energy production (Vacca et al., 2016; Valenti et al., 2018). We corroborate these findings within the Ts65Dn model *in vivo*, with reduction in ATP levels and overall mitochondrial RNA (*via Mt-Rnr1*) in Fr Ctx (**Figures 2C**, **3G**). We postulate significant decreases in trisomic mitochondrial RNA, as determined by *Mt-Rnr1* may drive gene expression changes seen in Fr Ctx tissue. Interestingly, these deficits may be the result of the use of regional tissue with admixed cell types for the protein assays, with the expectation that neuron-specific protein level changes may be obscured. Conversely, BF oxidative phosphorylation changes are likely to involve multiple neuronal subtypes within the BF and are able to be discriminated even in admixed tissue.

Mitochondrial dysfunction beyond the oxidative phosphorylation pathway has been linked to DS pathology including deficits in mitochondrial biogenesis, turnover, and mitophagy (Helguera et al., 2013; Izzo et al., 2018; Bordi et al., 2019; Mollo et al., 2019, 2020). The balance between biogenesis and mitophagy is perturbed using *in vitro* models of DS, with hyperactivation of proteins from the rapamycin (mTOR) pathway responsible for mitophagy and impaired activity of PGC-1α pathway responsible for biogenesis (Valenti et al., 2016; Bordi et al., 2019; Mollo et al., 2019, 2020). These studies link dysregulation of mitochondrial function to dysregulation of autophagy pathways, which have also been shown to be dysregulated in MSN BFCN neurons by single population RNA-seq (Alldred et al., 2021). We postulate BFCN degeneration in DS and AD may be directly related to the failure of mitochondrial turnover and mitophagy. From a translational

perspective, choline, an essential nutrient required for the production of the cholinergic neurotransmitter acetylcholine, the phosphatidylethanolamine N-methyltransferase (PEMT) pathway for generation of key substrates of neuronal membranes, and the primary methyl donor in the brain, requires properly functioning mitochondria and the oxidative phosphorylation pathway (Mailloux et al., 2016). Our collaborative group demonstrated metabolites of the PEMT pathway are significantly downregulated in the brains of Ts65Dn mice including within the BF and Fr Ctx (Yan et al., 2014), which also implicate deficits in mitochondria and the oxidative phosphorylation pathway. Further study on overall mitochondrial turnover, morphology, and mitophagy within *in vivo* DS and AD models are warranted.

RT-qPCR analysis and protein chemistry in trisomic BF and Fr Ctx reveals downregulation within oxidative phosphorylation genes and proteins that are specific to the brain regions analyzed. We recognize there are limitations and caveats to the studies we conducted using this approach. For example, each of these complexes in the oxidative phosphorylation pathway consists of numerous subunits, and the current interrogation was limited to one to two genes or proteins per complex. It is possible that other Complex I-V subunits will not be differentially regulated, although the larger number of changes identified by IPA and KEGG analysis from the MSN BFCN RNA-seq study of ∼6 MO Ts65Dn mice and 2N littermates analysis indicates BFCNs are significantly vulnerable and display an abundance of deficits in oxidative phosphorylation and mitochondrial function transcripts (Alldred et al., 2021). Another noted limitation is that when examining the correlation analysis between BF regional RT-qPCR and single population MSN BFCN RNA-seq, we were unable to compare *Mt-Nd4l* or *Mt-Atp8*, as they were not detected by RNA-seq (Alldred et al., 2021), likely due to the small size of the full-length mitochondrial RNA and low RNA input (McCormick et al., 2011; Stark et al., 2019). It is important to note this study was performed in male mice, and sex differences may exist in BFCN degenerative programs, as morphological differences between sexes in BFCNs have been demonstrated in trisomic mice (Kelley et al., 2014b). A cohort of female trisomic mice is currently being accrued for RNA-seq, RT-qPCR, and protein-based analyses, enabling sex differences to be evaluated in future studies. Importantly, Lanzillotta et al. (2021) demonstrate expression level changes in the oxidative phosphorylation pathway in Fr Ctx within trisomic mice during aging. Although beyond the scope of the present study, an aging time-course assessment of the Ts65Dn BF is warranted in future studies. Moreover, future assessments are planned to evaluate vulnerable cell types, brain regions, age, and sex in trisomic models in relation to parallel observations found in human postmortem DS and AD studies in the same cell types and regions.

In conclusion, select dysregulation of oxidative phosphorylation pathway members is found at the RNA and encoded protein levels within the vulnerable basocortical circuit in an established model of DS and AD at a timepoint where BFCN degeneration is occurring. Defects appear to initiate in the BF and travel in the synaptic pathway that connects this vulnerable region to a cortical terminal field associated with memory and executive function. These data suggest deficits in oxidative phosphorylation in the DS and possibly AD brain may be circuit driven, and more specific to vulnerable brain regions than previously appreciated, especially in the context of neuropathological disorders and age–related cognitive decline.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by IACUC Nathan Kline Institute.

AUTHOR CONTRIBUTIONS

MJA GES, and SDG designed the experiments. MJA performed experiments. SHL, MJA, and SDG performed analysis of data. MJA and SDG wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnagi.2021.707950/full#supplementary-material.

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Innate Immune System Activation and Neuroinflammation in Down Syndrome and Neurodegeneration: Therapeutic Targets or Partners?

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Innate immune system activation and inflammation are associated with and may contribute to clinical outcomes in people with Down syndrome (DS), neurodegenerative diseases such as Alzheimer's disease (AD), and normal aging. In addition to serving as potential diagnostic biomarkers, innate immune system activation and inflammation may play a contributing or causal role in these conditions, leading to the hypothesis that effective therapies should seek to dampen their effects. However, recent intervention studies with the innate immune system activator granulocyte-macrophage colony-stimulating factor (GM-CSF) in animal models of DS, AD, and normal aging, and in an AD clinical trial suggest that activating the innate immune system and inflammation may instead be therapeutic. We consider evidence that DS, AD, and normal aging are accompanied by innate immune system activation and inflammation and discuss whether and when during the disease process it may be therapeutically beneficial to suppress or promote such activation.

Keywords: innate immune system, inflammation, GM-CSF (granulocyte-macrophage colony-stimulating factor), Down syndrome, Alzheimer's disease, apolipoprotein E, drug repurposing and discovery, amyloid-β

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INTRODUCTION

Down syndrome (DS), most often caused by triplication of human chromosome 21 (Hsa21), is the most common genetic cause of both intellectual disability (ID) and age-associated cognitive decline (Epstein, 1990; Chapman and Hesketh, 2000; Silverman, 2007), affecting 1 in 700–1,000 live births worldwide (Centers for Disease Control and Prevention (CDC), 2006; Irving et al., 2008; Loane et al., 2013). The amyloid precursor protein (APP) plays a major role in the pathophysiology of Alzheimer's disease (AD), and because the *APP* gene resides on chromosome 21, its additional copy is primarily responsible for the fact that all people with DS develop AD brain pathology, including amyloid-β (Aβ) plaques and cerebral amyloid angiopathy, by age 40 (Epstein, 1990; Snyder et al., 2020). Additionally, adults with DS develop neurofibrillary tangles of hyperphosphorylated tau, oxidative stress, vascular abnormalities, and chronic neuroinflammation, which are pathologies also present in patients with AD or other neurodegenerative diseases (Wisniewski et al., 1985; Head et al., 2016; Snyder et al., 2020).

Despite substantial epidemiological, biochemical, and genetic evidence in support of the amyloid cascade hypothesis (Hardy, 2009), the AD pathogenic pathway can be modulated by other aspects of brain physiology, especially the innate immune system and neuroinflammation (Potter, 2001; El Khoury et al., 2007; Cribbs et al., 2012; Lambert et al., 2013; Bettcher et al., 2018; Barroeta-Espar et al., 2019; Taipa et al., 2019). Alois Alzheimer first suggested a potential role for inflammation in AD based on his observation of abnormal glial cells surrounding amyloid deposits (Alzheimer, 1907). The discovery that specific inflammatory proteins, such as the cytokine interleukin-1 (IL-1) and the inflammation/acute-phase protein α_1-antichymotrypsin (ACT), were upregulated in the AD brain and were associated with amyloid deposits solidified these early clues (Abraham et al., 1988; Mrak and Griffin, 2001; McGeer et al., 2006).

Inflammation is a complex multifactorial process in both the central nervous system (CNS) and the periphery, the activity of which varies depending on the disease stage. Microglia are the primary cell type associated with the innate immune system and neuroinflammation in the brain, with growing evidence suggesting that other cells, including astrocytes, neurons, oligodendrocytes, and pericytes also play significant roles, and brain inflammation in age-associated AD differs from that in DS-associated AD (Perry and Gordon, 1988; Colton and Wilcock, 2010; Wilcock et al., 2015). Thus, neuroinflammation may play a pivotal role in the development of AD (Akiyama et al., 2000), but the underlying mechanisms driving this pathological manifestation and its association with DS remain poorly understood.

In this mini-review, we first discuss the evidence that innate immune system activation and inflammation characterize both the CNS and the periphery. We will then review data that challenge the view that inflammation is solely detrimental, and instead suggest that both suppression and activation of the innate immune system and neuroinflammation may be beneficial, depending on the stage of the disorder. Finally, we will consider several new therapeutic strategies for regulating neuroinflammation, including the immune-modulatory cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF), inhibitors of apolipoprotein E (apoE), and microglial depletion *via* drugs that target the colony-stimulating factor-1 receptor (CSF1R).

Neuroinflammatory Biomarkers in CSF and Plasma from AD and DS

Studies have found increased cerebrospinal fluid (CSF) levels of immune biomarkers in mild cognitive impairment (MCI) and AD patients (Tarkowski et al., 2003; Galimberti et al., 2006; Jesse et al., 2009; Buchhave et al., 2010; Westin et al., 2012; Kauwe et al., 2014; Counts et al., 2017; Whelan et al., 2019). Peripheral immune cells, such as neutrophils, monocytes, and lymphocytes, also produce (and respond to) inflammatory cytokines, which are significantly upregulated in the blood of AD patients and may also be derived from the CNS (Kim et al., 2008; Diniz et al., 2010; Morgan et al., 2019). Similar increases in blood levels of immune biomarkers are found in people with DS (Petersen and O'Bryant, 2019; Huggard et al., 2020a,b). One inflammation-based hypothesis is that people with DS are in a state of chronic abnormal inflammation, including features of auto-inflammation, across the lifespan that influences all phenotypes and disease risk. Specifically, proteomic analyses of plasma and brain tissue from people with DS revealed dysregulation of inflammatory protein expression, including increases in several pro-inflammatory cytokines, and decreases in numerous complement cascade components (Sullivan et al., 2016, 2017; Zhang et al., 2017). Another study revealed elevated levels of both pro-inflammatory and anti-inflammatory cytokines in plasma from children with DS (Huggard et al., 2020a,b). Activation of astrocytes and microglia, the secretion of inflammatory cytokines (e.g., IL-1, IL-6, and TNFα), and acute phase proteins are observed in both the brains and blood of people with DS, indicating an "inflammatory endophenotype" (Petersen and O'Bryant, 2019). In a cross-sectional analysis of people with DS, plasma glial fibrillary acidic protein (GFAP), a marker of astrogliosis, was found to increase starting in their mid-40s (Hendrix et al., 2021).

Timing and Effects of Innate Immune System Activation and Neuroinflammation in DS and AD

Epidemiological studies suggest that elevated immune biomarkers in the blood may be evident years prior to the manifestation of clinical symptoms of AD or AD-related dementias (ADRDs) in the typical population (Schmidt et al., 2002; Ridolfi et al., 2013; Leszek et al., 2016; Busse et al., 2017; Wendeln et al., 2018). Higher plasma levels of GFAP are correlated with lower measures of episodic memory and microstructural integrity in AD, MCI, and also in healthy aged donors (Bettcher et al., 2021). As discussed, people with DS also have increasing levels of plasma GFAP starting in their mid-40s (Hendrix et al., 2021). Thus, although initially thought to be a secondary effect of aberrant protein accumulation, changes in the innate immune system and neuroinflammation are now thought to be a core, early feature of both DS and AD that interface with, and may contribute to, clinical manifestations of cognitive disorders and decline (Lucin and Wyss-Coray, 2009; Heneka et al., 2015).

Hsa21 harbors numerous innate immune system and neuroinflammation-associated genes that are therefore triplicated in most people with DS (**Table 1**). Notably, four genes encoding interferon receptors reside on Hsa21, and interferon-related signaling is upregulated in people with DS (Sullivan et al., 2016; Araya et al., 2019; Powers et al., 2019). The inflammatory response microRNA miR-155 also resides on Hsa21 and is overexpressed in DS (Guo et al., 2019). Proteomic analyses have revealed a striking increase in both pro- and anti-inflammatory cytokines in plasma and brain tissue samples from people of all ages with DS (Sullivan et al., 2017; Zhang et al., 2017; Flores-Aguilar et al., 2020; Huggard et al., 2020b) and in mouse models of DS (Ahmed et al., 2012, 2013; Spellman et al., 2013; Block et al., 2015), which express homologs of Hsa21-encoded inflammation-related genes (**Table 1**). Together, these findings have led to the hypothesis that DS inherently results

TABLE 1 | A summary of inflammation-related genes located on HSA21.

Gene	Protein	Function	References	Present in common DS mouse models[#]	AD risk identified in GWAS[@]
ABCG1	ATP binding cassette subfamily G member 1	Catalyzes phospholipid and cholesterol efflux and maintains macrophages in an anti-inflammatory state.	Wojcik et al. (2008)	Dp17, Tc1	Wollmer et al. (2007) and Beecham et al. (2014)
ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif 1.	Secreted protease is known to be induced by IL-1β.	Kuno et al. (1997)	Dp16, Ts65Dn, Tc1	Kunkle et al. (2019), Niu et al. (2019), and Tan et al. (2021)
ADAMTS5	ADAM metallopeptidase with thrombospondin type 1 motif 5.	Secreted protease known to be induced by IL-1β and TGFβ.	Yamanishi et al. (2002)	Dp16, Ts65Dn, Tc1	none
APP	Amyloid beta precursor protein	Neuronal acute phase protein precursor of Aβ fragments in Alzheimer's plaques and inducer of IL-1β.	Glenner and Wong (1984), Tanzi et al. (1988), and Barger and Harmon (1997)	Dp16, Ts65Dn, Tc1*	Guyant-Maréchal et al. (2007), Nowotny et al. (2007), and Lv et al. (2008)
BACE2	Beta-secretase 2	Cleaves APP for less Aβ and increases IL-1R2, a decoy proteinfor excess IL-1 capture.	Kuhn et al. (2007)	Dp16, Ts65Dn, Tc1	Myllykangas et al. (2005)
CBS	Cystathionine beta-synthase	Catalyzes production of hydrogen sulfide bimodalregulation of inflammation.	Sen et al. (2011)	Dp17, Tc1	Beyer et al. (2004)
CSTB	Cystatin B	Thiol protease inhibitor involved in Aβ clearance.	Yang et al. (2011) and Maher et al. (2014)	Dp10, Tc1	Kurt et al. (2020)
CXADR	CXADR Ig-like cell adhesion molecule	Activation of JNK and p38-MAPK pathways leading toproduction of M1 cytokines.	Yuen et al. (2011)	Dp16, Tc1	none
DYRK1A	Dual specificity tyrosine phosphorylation regulated kinase 1A	Serine/threonine and tyrosine kinase that regulates the NFκB pathway and phosphorylates tau.	Latour et al. (2019)	Dp16, Ts65Dn, Tc1	Kimura et al. (2007)
IFNAR1	Interferon alpha and beta receptor subunit 1	Activates JAK/STAT mediated anti-inflammatory pathway.	Kim et al. (1997)	Dp16, Ts65Dn	Patel et al. (2021)
IFNAR2	Interferon alpha and beta receptor subunit 2	Activates JAK/STAT mediated anti-inflammatory pathway.	Kim et al. (1997) and Boselli et al. (2010)	Dp16, Ts65Dn	none
IFNGR2	Interferon gamma receptor 2	Activates JAK/STAT mediated anti-inflammatory pathway.	Boselli et al. (2010)	Dp16, Ts65Dn	none
PRMT2	Protein arginine methyltransferase 2	Blocks the actions of NFκB in the nucleus.	Ganesh et al. (2006)	Dp10, Tc1[$]	none
RCAN1	Regulator of calcineurin 1	Inhibits calcineurin-dependent transcription and is regulated by STAT2.	Lee et al. (2012)	Dp16, Ts65Dn	Lin et al. (2011)
RIPK4	Receptor interacting serine/threonine kinase 4	Necessary for signaling through TNF receptor 1.	Rountree et al. (2010)	Dp16, Ts65Dn, Tc1	none
RUNX1	RUNX family transcription factor 1	Transcription factor regulating T-cell function.	Tang et al. (2018)	DP16, Ts65Dn, Tc1[&]	Kimura et al. (2007) and Patel et al. (2011)
S100B	S100 calcium binding protein B	Upregulates IL-1β and APP expression, released in responseto TNFα.	Li et al. (1998), Liu et al. (2005), and Donato et al. (2013)	Dp10, Tc1[$]	Lambert et al. (2007)

(Continued)

TABLE 1 | Continued

Gene	Protein	Function	References	Present in common DS mouse models[#]	AD risk identified in GWAS[@]
SOD1	Superoxide dismutase 1	Scavenges superoxide radicals producing H_2O_2 and O_2.	Danciger et al. (1986)	Dp16, Ts65Dn, Tc1	none
TIAM1	TIAM Rac1 associated GEF 1	Necessary for cytokine-mediated generation of oxidativespecies through NADPH oxidase.	Subasinghe et al. (2011)	Dp16, Ts65Dn, Tc1	none

Some parts of ***Table 1*** *are reproduced from Wilcock and Griffin (2013) with permission under the CC BY 2.0 license (http://creativecommons.org/licenses/by/2.0/), Copyright © Wilcock and Griffin (2013); licensee BioMed Central Ltd. Abbreviations: Aβ, β-amyloid; ADAM, a disintegrin and metalloprotease; APP, amyloid beta precursor protein; ATP, adenosine triphosphate; CXADR, coxsackievirus and adenovirus receptor; DS, Down syndrome; IL, interleukin; GEF, guanine nucleotide exchange factor; GWAS, genome-wide associate study; MAPK, mitogen-activated protein kinase; NADPH, Nicotinamide adenine dinucleotide phosphate; RUNX, Runt-related transcription factor; TNF, tumor necrosis factor; TGF, transforming growth factor; JAK/STAT, Janus kinase signal transducer and activator of transcription; JNK, c-Jun N-terminal kinase; TIAM, T-cell lymphoma invasion and metastasis; TNFR, tumor necrosis factor receptor; NFκB, Nuclear factor-kappa B; STAT2, signal transducer and activator of transcription 2. [#]Tc1 mice express one copy of the human gene and two copies of the homologous mouse gene, while Ts65Dn, Dp10, Dp16, and Dp17 mice express three copies of the homologous mouse gene, as identified in Ahmed et al. (2013). *In Tc1 mice, the APP gene is re-arranged, and therefore human APP is not functionally expressed. [$]In Tc1 mice, the human PRMT2 and S100B genes are duplicated, in addition to two copies of the homologous mouse genes. [&]In Tc1 mice, the human RUNX1 gene is partially deleted. [@]Some relevant GWAS studies were identified using the AlzGene database (Bertram et al., 2007).*

in chronic neuroinflammation, including auto-inflammation and astrogliosis (Sullivan et al., 2016; Rachubinski et al., 2019; Snyder et al., 2020). Although non-steroidal anti-inflammatory drugs (NSAIDs) were not therapeutic for AD, it is possible that more selective blockers of the innate immune system might be effective in AD and/or DS, for example, through inhibition of the TLR2-MyD88 interaction or of JAK-1 (Rangasamy et al., 2018; Rachubinski et al., 2019; Tuttle et al., 2020).

Apolipoprotein E in Inflammation and Neurodegeneration in DS and AD

Inheritance of the *APOE* ε4 allele (*APOE4*) is the strongest risk factor for AD, besides age, with one copy of *APOE4* leading to a three-fold increased risk of AD and two copies leading to a 15-fold increased risk of AD (Corder et al., 1993; Strittmatter et al., 1993; Strittmatter and Roses, 1995). Inheritance of the *APOE4* allele also significantly increases the risk for cognitive decline and dementia in middle-aged people with DS (Rubinsztein et al., 1999; Deb et al., 2000), and it increases the risk of mortality by five-fold compared to non-*APOE4* carriers (Zigman et al., 2005). Furthermore, brain *APOE* expression is significantly upregulated in people with DS compared to the typical population (Lockstone et al., 2007), which might exacerbate DS-associated AD.

Similar to the inflammation/acute-phase protein ACT, apoE is also associated with amyloid deposits (Wisniewski and Frangione, 1992; Wisniewski et al., 1993; Ma et al., 1994; Sanan et al., 1994; Wisniewski et al., 1994). Although not typically considered a neuroinflammatory molecule, *APOE* expression is upregulated by astrocytes and microglia early in the AD pathological process (Keren-Shaul et al., 2017; Krasemann et al., 2017; Kang et al., 2018; Rangaraju et al., 2018), and it also plays a pivotal role in modulating the neuroinflammatory cascade by both Aβ-dependent and Aβ-independent pathways (McGeer et al., 1997; Maezawa et al., 2006; Zhu et al., 2012; Cudaback et al., 2015; Shi et al., 2017; Lin et al., 2018).

Increasing evidence shows that apoE plays a role in amyloid formation by promoting the aggregation of the Aβ peptide to form insoluble filaments, thereby also inhibiting the clearance of Aβ from the brain (Potter and Wisniewski, 2012; **Figure 1**). For example, apoE4 catalyzes the formation of neurotoxic Aβ oligomers and fibrils (Ma et al., 1994, 1996; Sanan et al., 1994; Wisniewski et al., 1994; Castano et al., 1995; Golabek et al., 1996; Soto et al., 1996; Manelli et al., 2007; Cerf et al., 2011; Hashimoto et al., 2012; Koffie et al., 2012; Liu et al., 2017). Fortunately, this mechanistic pathway lends itself to therapeutic approaches targeting apoE using antibodies, antisense oligonucleotides, or structural correctors (Brodbeck et al., 2011; Chen et al., 2012; Liao et al., 2014; Huynh et al., 2017; Wang et al., 2018; Xiong et al., 2021), or by targeting the interaction between apoE and Aβ using peptide blockers or small molecule drugs (Ma et al., 1996; Sadowski et al., 2004; Pankiewicz et al., 2014; Johnson et al., 2021a). Recently, high-throughput screens from our lab have identified several Food and Drug Administration (FDA)-approved drugs, including the anti-depressant drug imipramine and the anti-psychotic drug olanzapine, that inhibit the apoE-Aβ interaction and appear to improve cognition in AD patients, and especially in *APOE4* carriers, in our retrospective analyses of human clinical data (Johnson et al., 2021a).

GM-CSF as a Neuroinflammatory Modulator in AD

Patients with rheumatoid arthritis (RA) have a three- to eight-fold reduced risk of developing AD, suggesting a potential role for inflammation and the innate immune system in AD (McGeer et al., 1996). Although the reduced AD risk in patients with RA was initially attributed to their frequent use of NSAIDs (McGeer et al., 1996), NSAID treatment showed no benefit in clinical trials of either AD or MCI patients (McGeer et al., 2006; ADAPT_FS_Research_Group, 2015).

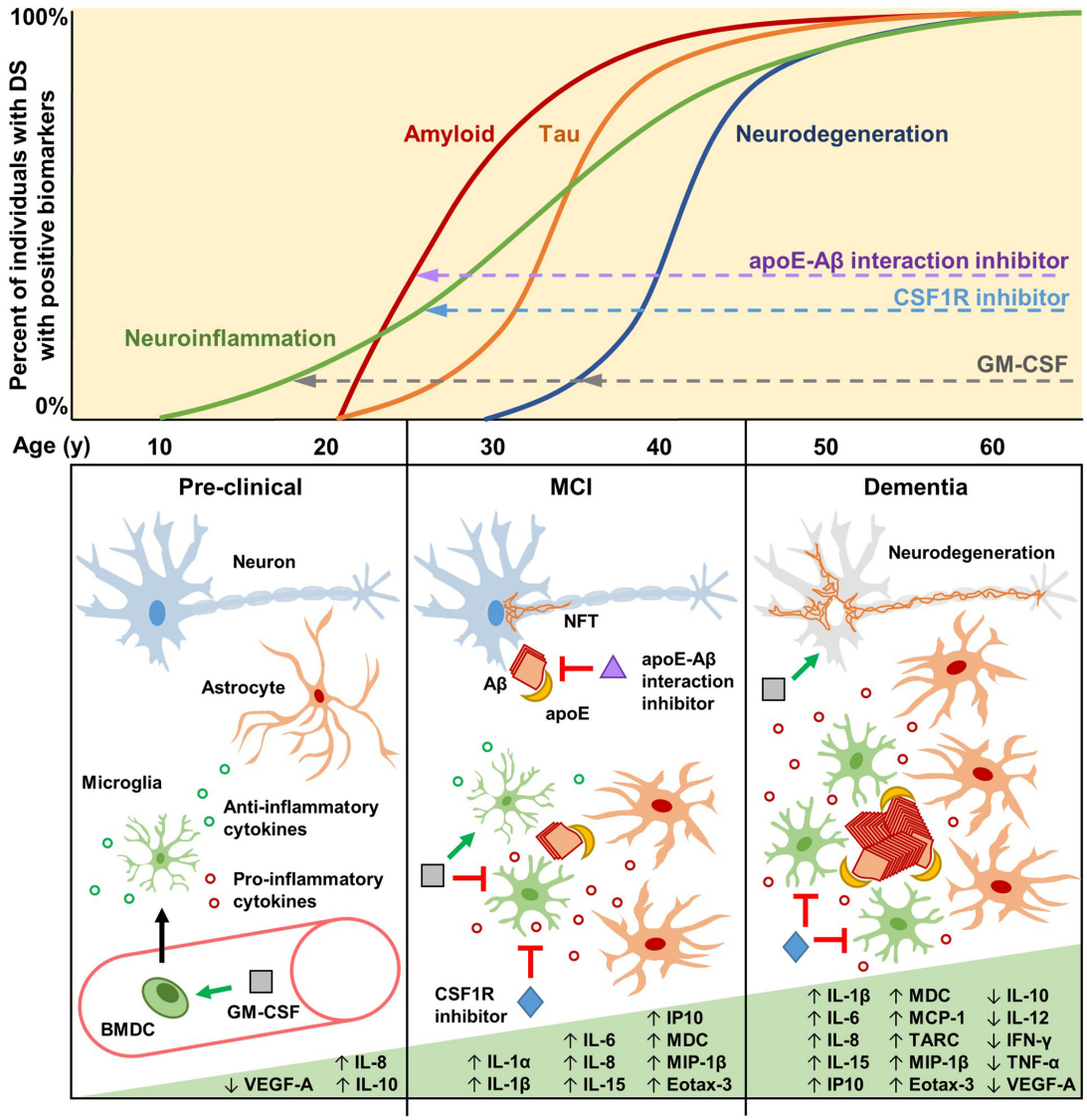

FIGURE 1 | Biomarkers, cytokines, and potential therapeutic mechanisms for neuroinflammation in Down syndrome (DS) and Alzheimer's disease (AD). Biomarkers of neuroinflammation, amyloid and tau pathology, and neurodegeneration increase over the lifetimes of the vast majority of individuals with DS. Neuroinflammatory biomarkers such as GFAP may begin increasing as early as the late teenage years and progressively increase throughout life (Hendrix et al., 2021). Changes in amyloid biomarkers are detectable after 20 years of age, with *APOE* genotype significantly contributing to risk (Rubinsztein et al., 1999; Deb et al., 2000), likely due to apoE-catalyzed amyloid-β (Aβ) polymerization during the early seeding stages of amyloid formation (Potter and Wisniewski, 2012). Tau pathology is detectable soon after amyloid forms, but it accumulates more slowly. By the age of 40, every individual with DS has the hallmark amyloid and tau neuropathology of AD. Biomarkers of neurodegeneration, such as changes in brain volume and glucose metabolism, are identifiable after 30 years of age, proceeding and generally correlating well with tau pathology. During the pre-clinical stage, GM-CSF may have therapeutic potential by modulating the immune/neuroinflammatory cascade in order to prevent and/or delay amyloidogenesis. GM-CSF stimulates bone marrow-derived cells (BMDCs) to mobilize and extravasate to the brain, or GM-CSF may enter the brain directly, where the resulting activated microglia modulate their cytokine expression and reactivity (Bhattacharya et al., 2015a; Abe et al., 2020). During the mild cognitive impairment (MCI) stage, preceding clinical dementia, Aβ aggregates begin to form, catalyzed by apoE, which then induces intraneuronal tau hyperphosphorylation and neurofibrillary tangle (NFT) formation. Therapeutic molecules that can inhibit the apoE-Aβ interaction (e.g., Sadowski et al., 2004; Pankiewicz et al., 2014; Johnson et al., 2021a) may significantly reduce amyloid deposition, thus preventing/reducing downstream pathologies. Activated microglia and astrocytes, characterized by increased soma size and shortened processes, cluster around amyloid plaques and increase the expression of interleukins and other pro-inflammatory cytokines. At this stage, GM-CSF may act to maintain microglia in a non-activated state, promote anti-inflammatory cytokine expression, and reduce pro-inflammatory cytokine expression (Ahmed et al., 2021; Potter et al., 2021). CSF1R inhibitors may also be beneficial at this stage to reduce the numbers of activated microglia while maintaining quiescent microglia that play important roles in immune surveillance and brain homeostasis (Johnson et al., 2021b). The clinical dementia stage is characterized by neurodegeneration due to widespread NFT formation and chronic neuroinflammation that persists through the mid-40s, 50s, and 60s, during which a majority of individuals with DS have clinical dementia. Some inflammatory markers are reported to decrease during this stage, possibly due to cellular exhaustion and degeneration (Flores-Aguilar et al., 2020). GM-CSF may therapeutically modify neurodegeneration *via* neuroprotective effects, while CSF1R inhibitors may reduce the numbers and effects of chronically activated microglia.

As an alternative, we hypothesized that intrinsic factors associated with RA pathogenesis itself may underlie its AD protective effect(s). We identified GM-CSF as one such factor that is upregulated in the blood of RA patients and found that subcutaneous injection of GM-CSF for 20 days increased microglial activation, reduced amyloid pathology by more than 50%, and completely reversed the cognitive impairment of transgenic AD mice (Boyd et al., 2010), which has been replicated by other groups (Castellano et al., 2017; Kiyota et al., 2018). Treatment with recombinant human GM-CSF (sargramostim/Leukine®) was also associated with improved cognition in cancer patients undergoing hematopoietic stem cell transplantation (Jim et al., 2012). These findings led us to design and carry out a placebo-controlled, randomized, double-blind Phase II clinical trial in mild-to-moderate AD participants, which showed that subcutaneous injection of sargramostim (5 days/week for 3 weeks) was safe, associated with reduced plasma biomarkers of neuronal damage/neurodegeneration (i.e., total tau and UCH-L1), and improved cognition based on Mini-Mental State Examination (MMSE) scores (Potter et al., 2021). These findings suggest that during the early stages of AD, activation of the innate immune system may be beneficial, which has now led to a longer–term trial.

GM-CSF as an Amyloid-Independent Cognition Enhancer in DS and Normal Aging

People with DS have significant ID throughout their 60-year life expectancy (Bittles et al., 2007), with no treatments available. Several drugs have been tested in mouse models of DS, primarily in Ts65Dn mice, with some promising results showing the rescue of DS-related cognitive deficits. Unfortunately, no such drugs have shown significant benefits in clinical trials of people with DS (reviewed in Gardiner, 2015; Vacca et al., 2019).

Because GM-CSF treatment improved cognition and reduced amyloid in mouse models of AD, we investigated its effects in the Dp16 mouse model of DS. Our findings show that in both Dp16 mice and their wild-type (WT) littermates, GM-CSF treatment improves learning/memory in the radial arm water maze, a hippocampal-based task. In Dp16 mice, GM-CSF treatment also ameliorates the abnormal astrocyte morphology and aggregation and partially normalizes the levels of interneurons (Ahmed et al., 2021). Although GM-CSF treatment evidently improves learning/memory in mouse models of AD by removing amyloid plaques in the brain, it is noteworthy that WT mice and mouse models of DS, including Dp16 mice, do not develop AD amyloid pathology at any age. Therefore, GM-CSF treatment must lead to improved learning/memory in WT and Dp16 mice *via* an amyloid-independent mechanism(s), likely related to its pro-inflammatory or inflammation-modulating activity.

Despite its known pro-inflammatory properties, GM-CSF also affects multiple CNS processes that are consistent with, and provide some insights into, its unexpected beneficial effects on learning/memory in a mouse model of DS. Specifically, GM-CSF promotes recovery from neuronal damage or dysfunction in animal models of stroke (Schneider et al., 2007; Schäbitz et al., 2008; Kong et al., 2009; Theoret et al., 2016), traumatic brain injury (Shultz et al., 2014; Kelso et al., 2015), and acute retinal ganglion cell injury (Schallenberg et al., 2012; Legacy et al., 2013). GM-CSF can cross the blood-brain barrier (McLay et al., 1997) and is also produced within the brain, where numerous cell types express the GM-CSF receptor, including neurons, oligodendrocytes, microglia, astroglia, and endothelial cells, which would allow for both paracrine and autocrine signaling (Baldwin et al., 1993; Sawada et al., 1993).

Many studies have shown that both people with DS and typical aging adults exhibit an auto-inflammatory or "inflammaging" syndrome (Trollor et al., 2012; Frasca and Blomberg, 2016; Ashraf-Ganjouei et al., 2020; Serre-Miranda et al., 2020) that might predict that GM-CSF treatment would be detrimental. However, GM-CSF is not merely a pro-inflammatory molecule. A more accurate description is that GM-CSF modulates the innate immune system, especially in the setting of immune system dysregulation in the periphery and in the brain (Boyd et al., 2010; Bhattacharya et al., 2015a,b; Borriello et al., 2019). Indeed, GM-CSF treatment not only increases the levels of many cellular and cytokine biomarkers of inflammation in the blood of AD patients (e.g., neutrophils, monocytes, lymphocytes, IL-2, IL-6, and TNFα), but also reduces the levels of the inflammatory cytokine IL-8 and increases the levels of the typically anti-inflammatory cytokine IL-10 (Potter et al., 2021). Thus, GM-CSF has a much more complex physiological effect than simply being pro-inflammatory. Furthermore, suppressing the inflammation associated with DS in the periphery may be beneficial in the setting of certain acute disorders (Rachubinski et al., 2019). Thus, growing evidence highlights the complexity of the innate immune system in the context of inflammation in people with DS.

Notably, the fact that we observed improved memory in WT aging mice treated with GM-CSF (Boyd et al., 2010; Ahmed et al., 2021) suggests that GM-CSF has a cognition/memory enhancing activity that is independent of disease and suggests that modulation of the innate immune system may help prevent normal age-related memory decline. In contrast to previous expectations that inhibiting inflammation and the innate immune system would be the most effective therapy for co-morbidities of DS, the beneficial effects of GM-CSF on learning and memory may reflect its stimulating pro-inflammatory activity, its other physiological/cellular effects, or both (**Figure 1**).

Therapeutic Modulation of Microglial Numbers and Activation

It is becoming increasingly clear that microglia play multiple, and often disparate, roles at different stages of the innate immune and neuroinflammatory responses in DS and in AD. Accordingly, microglial reduction/depletion has also been investigated as a therapeutic approach to DS, AD, and other neurodegenerative diseases. For example, small molecule drugs targeting CSF1R, which is crucial for microglial proliferation

and survival, have been repurposed from cancer indications (Cannarile et al., 2017) and used to modulate microglial levels in the CNS (**Figure 1**). Indeed, microglial reduction *via* CSF1R inhibition was found to rescue several cognitive deficits in Dp16 mice (Pinto et al., 2020). In AD mouse models, microglial depletion prior to amyloid deposition was shown to be critical for therapeutic efficacy (Sosna et al., 2018; Spangenberg et al., 2019; Son et al., 2020). Likewise, in mouse models of primary tauopathy, characterized by inclusions of the protein tau in neural cells (Kovacs, 2015), CSF1R inhibitors reduced pathological tau aggregation and subsequent neurodegeneration (Mancuso et al., 2019; Shi et al., 2019). However, recent evidence suggests that complete microglial depletion is neither necessary nor desirable for extending lifespan in tauopathy mice and that microglia resilient to CSF1R inhibition exist in a quiescent, non-activated state and may serve important roles in prevention and recovery from tau-induced neurodegeneration (Johnson et al., 2021b). Together, these studies underscore the importance of carefully considering microglial state and function over the disease course in order to appropriately balance microglial stimulation and repression therapeutically.

CONCLUSION

Collectively, the recent data indicate that targeted enhancement or inhibition of the innate immune system and inflammatory cytokines can effectively treat DS, AD, and normal aging. These findings provide compelling evidence that the long-held belief that inflammation and innate immune system activation primarily play negative roles in DS, AD, and normal aging must be reassessed. Although GM-CSF is the first modulatory cytokine to exhibit therapeutic potential in inflammation-associated disorders, it serves as a proof-of-principle, and other therapeutic molecules could use a similar approach. Furthermore, combination therapies that pair GM-CSF with small molecule inhibitors of apoE or CSF1R at appropriate neuroinflammatory stages may be particularly effective for treating people with DS and/or AD.

AUTHOR CONTRIBUTIONS

All authors contributed to the design and writing of the manuscript. All authors contributed to the article and approved the submitted version.

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Maternal Choline Supplementation as a Potential Therapy for Down Syndrome: Assessment of Effects Throughout the Lifespan

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Maternal choline supplementation (MCS) has emerged as a promising therapy to lessen the cognitive and affective dysfunction associated with Down syndrome (DS). Choline is an essential nutrient, especially important during pregnancy due to its wide-ranging ontogenetic roles. Using the Ts65Dn mouse model of DS, our group has demonstrated that supplementing the maternal diet with additional choline (4-5× standard levels) during pregnancy and lactation improves spatial cognition, attention, and emotion regulation in the adult offspring. The behavioral benefits were associated with a rescue of septohippocampal circuit atrophy. These results have been replicated across a series of independent studies, although the magnitude of the cognitive benefit has varied. We hypothesized that this was due, at least in part, to differences in the age of the subjects at the time of testing. Here, we present new data that compares the effects of MCS on the attentional function of adult Ts65Dn offspring, which began testing at two different ages (6 vs. 12 months of age). These data replicate and extend the results of our previous reports, showing a clear pattern indicating that MCS has beneficial effects in Ts65Dn offspring throughout life, but that the magnitude of the benefit (relative to non-supplemented offspring) diminishes with aging, possibly because of the onset of Alzheimer's disease-like neuropathology. In light of growing evidence that increased maternal choline intake during pregnancy is beneficial to the cognitive and affective functioning of all offspring (e.g., neurotypical and DS), the addition of this nutrient to a prenatal vitamin regimen would be predicted to have population-wide benefits and provide early intervention for fetuses with DS, notably including babies born to mothers unaware that they are carrying a fetus with DS.

Keywords: Down syndrome, maternal choline supplementation, aging, learning, attention, septohippocampal circuit

INTRODUCTION

Down syndrome (DS) is the most common genetic cause of intellectual disability, characterized by deficits in language comprehension and production, as well as impairments of learning, memory, and various executive functions (e.g., planning, inhibitory control, and attention) (Chapman and Hesketh, 2000; Rachidi and Lopes, 2010). In addition, nearly all individuals with DS develop the neuropathological changes associated with Alzheimer's disease (AD) by the third to fourth decade of life, including senile plaques, neurofibrillary tangles, and degeneration of basal forebrain cholinergic neurons (BFCNs) (Wisniewski et al., 1985a,b; Mann et al., 1986; Lai and Williams, 1989; Leverenz and Raskind, 1998; Hartley et al., 2015). There are currently no effective therapies to prevent or ameliorate the cognitive impairment and brain pathology associated with DS, so the identification of interventions is of paramount importance.

Several mouse models have been generated that recapitulate the hallmark phenotype of humans with DS, providing translational tools to elucidate the pathogenic processes associated with this disorder and the testing of potential therapies. The Ts65Dn mouse model is well-characterized and replicates key aspects of human DS neuropathology and associated behavioral deficits (Davisson et al., 1990; Reeves et al., 1995; Holtzman et al., 1996; Hyde and Crnic, 2001; Hyde et al., 2001a,b; Bimonte-Nelson et al., 2003; Hunter et al., 2003). Notably, like humans with DS, Ts65Dn mice exhibit impairments in hippocampal-dependent learning and memory, attentional dysfunction, hyperactivity, and heightened emotionality (Granholm et al., 2000; Hyde et al., 2001b; Driscoll et al., 2004; Moon et al., 2010; Velazquez et al., 2013; Ash et al., 2014; Powers et al., 2016, 2017, 2018). Also similar to humans with DS, Ts65Dn mice are born with intact BFCNs, and projections from these neurons to the hippocampus (septohippocampal circuit) and neocortex (basocortical circuit) show normal organization. But, similar to the early-onset degeneration of these projection systems seen in adult humans with DS, Ts65Dn mice display progressive BFCN atrophy beginning at about 6 months of age (MO) (Holtzman et al., 1996; Granholm et al., 2000; Cooper et al., 2001), coincident with a progressive decline in cognitive functioning (Hyde and Crnic, 2001).

Our group has utilized the Ts65Dn mouse model to test the hypothesis that maternal choline supplementation (MCS) improves cognitive and affective functioning in DS, as well as protects against age-related degeneration of BFCNs that give rise to the septohippocampal and basocortical connectomes. Choline is an essential nutrient, and its availability during pregnancy is a key factor in fetal neurodevelopment (Zeisel, 2006). Choline is a precursor for phosphatidylcholine and sphingomyelin, two critical components of neuronal and non-neuronal lipid membranes, and it is essential for the biosynthesis of acetylcholine, a neurotransmitter that regulates neuronal proliferation, differentiation, migration, plasticity, and synapse formation (Lauder and Schambra, 1999; Abreu-Villaça et al., 2011; Bernhard et al., 2019). Choline is also the primary dietary source of methyl groups, thereby contributing to the epigenetic regulation of gene expression *via* DNA and histone methylation (Kovacheva et al., 2009; Blusztajn and Mellott, 2012; Jiang et al., 2012). Because of the wide-ranging ontogenetic roles of choline, dietary demand is markedly increased during pregnancy, when there is a pronounced depletion of maternal choline stores throughout gestation (Yan et al., 2012; Jiang et al., 2014). Importantly, numerous studies using normal rodents have demonstrated that increasing maternal intake of choline during critical periods of early development has lasting beneficial effects on cognitive and affective functioning of offspring. Specifically, supplementing the maternal diet with additional choline during pregnancy (approximately four times higher than standard lab chow) has been shown to improve memory, spatial cognition, and attentional function of offspring (Meck and Williams, 2003; McCann et al., 2006), and also emotion regulation (Cheng et al., 2008)—all domains which are adversely affected in DS.

Over the past 10 years, we have demonstrated that supplementing the diet of Ts65Dn dams during pregnancy and lactation with additional choline is beneficial to trisomic offspring (Moon et al., 2010; Velazquez et al., 2013; Ash et al., 2014; Powers et al., 2017). Specifically, supplemented trisomic mice (vs. non-supplemented counterparts) exhibited significant improvements in spatial cognition, attention, and emotion regulation (Moon et al., 2010; Velazquez et al., 2013; Ash et al., 2014). These same studies also revealed that MCS normalizes adult hippocampal neurogenesis in the trisomic offspring and prevents degeneration of BFCNs of the medial septum. Moreover, the behavioral indices of spatial cognition correlated significantly with both hippocampal neurogenesis and medial septal cholinergic neuron density, suggesting that they contribute to the functional benefits. We have also shown that MCS normalizes the expression of genes associated with synaptic plasticity, calcium signaling, and neurodegeneration in the septohippocampal circuit (Alldred et al., 2018, 2019), providing plausible underlying mechanisms of neurodegeneration.

Although benefits of MCS for Ts65Dn mice have been replicated across several studies (Moon et al., 2010; Velazquez et al., 2013; Ash et al., 2014; Powers et al., 2017), it is notable that the magnitude of improvement in attentional function has varied across studies, for reasons that we are only now beginning to understand. Specifically, a robust benefit of MCS was observed in trisomic offspring when attentional function was assessed between 6 and 10 MO (Moon et al., 2010). By contrast, when testing occurred between 12 and 17 MO, a benefit of MCS for trisomic offspring was seen for spatial cognition, hippocampal neurogenesis, and neuroprotection of BFCNs, but was less pronounced for attention (Velazquez et al., 2013; Ash et al., 2014; Powers et al., 2017). The factors underlying these discrepancies were unclear since the housing facility, testing equipment, personnel, and age at which the attentional studies were performed differed.

Here, we present new data from two investigations, which strongly indicate that the age of subjects at the time of testing was likely responsible for these differences in outcome. In the first study (Study 1 herein), we initiated behavioral testing of the offspring at 12 MO, so that gene expression and other neurologic endpoints could be compared and contrasted with our prior neurological findings (Velazquez et al., 2013; Ash

et al., 2014; Powers et al., 2017). Here, we did not observe significant benefits of MCS on attentional function; these data were similar to the modest effects of MCS that we reported previously (Powers et al., 2017), when the offspring were tested at this older age. These findings led to the hypothesis that the reduced magnitude of the attentional benefit was due to the older age of subjects at the time of testing. To test this hypothesis, we conducted another study, which initiated attention testing at 6 MO (Study 2 herein). As observed in our original report on MCS in Ts65Dn mice of this same age (Moon et al., 2010), we found robust improvement in attentional function in the choline-supplemented trisomic offspring, relative to non-supplemented trisomics. Together these four studies lend support to our hypothesis that attentional benefits of MCS occur during early- and mid-life in trisomic offspring, but diminish with aging.

METHODS (STUDIES 1 AND 2)

The methods for both studies are identical other than the age at which behavioral testing was initiated. Thus, the following methods pertain to both studies, unless otherwise noted.

Subjects

Breeder pairs (female Ts65Dn and male C57Bl/6J Eicher × C3H/HeSnJ F1 mice) were purchased from Jackson Laboratories (Bar Harbor, Maine) and mated at Cornell University, Ithaca, New York, USA. All dams are trisomic but give birth to both trisomic and normal disomic (2N) mice. Upon arrival, breeder pairs were randomly assigned to receive either a standard purified rodent diet (containing choline) or one supplemented with additional choline. The control diet was AIN-76A purified rodent diet containing 1.1 g/kg choline chloride, whereas the choline-supplemented diet was the same (AIN-76A purified rodent diet) but containing 5.0 g/kg choline chloride (Dyets Inc., Bethlehem, Pennsylvania, USA). The control diet supplies adequate choline as recommended by the National Research Council, Nutrient Requirements of Laboratory Animals, Fourth Revised Edition [National Research Council (US) Subcommittee on Laboratory Animal Nutrition, 1995]. The choline-supplemented diet provided ∼4.5 times the concentration of choline, within the range of dietary variation observed in humans (Detopoulou et al., 2008). Breeder pairs were provided *ad libitum* access to water and their assigned diets, and remained on this diet throughout the prenatal and postnatal periods. Standard cages contained paper bedding and several objects (e.g., plastic igloo, *t*-tube, plastic-gel bone, and cotton square). Mice were maintained on a 12-h light-dark cycle under temperature- and humidity-controlled conditions.

Offspring were weaned on postnatal day (PND) 21, and all subjects were provided *ad libitum* access to water and the control diet. Thus, choline supplementation was provided only maternally, starting before conception and ending on PND 21. Ear punch tissue was sent to Jackson Laboratories for genotyping by quantitative polymerase chain reaction for the detection of the extra chromosomal segment and determination of *Pde6Brd1* homozygosity, a recessive mutation leading to retinal degeneration (Bowes et al., 1993). *Pde6Brd1* homozygous mice were excluded from the study.

For each study, we used male mice ($n = 16$) comprising four groups: (i) disomic offspring of dams on the control diet (**2N**); (ii) Ts65Dn offspring of dams on the control diet (**Ts**); (iii) disomic offspring of dams on the choline-supplemented diet (**2N+**); and (iv) Ts65Dn offspring of dams on the choline-supplemented diet (**Ts+**). There was some attrition due to animal health concerns or the failure to perform the basic training task. The final subject numbers included in statistical analyses in Study 1 were: 2N ($n = 14$), 2N+ ($n = 16$), Ts ($n = 15$), and Ts+ ($n = 15$). In Study 2: 2N ($n = 13$), 2N+ ($n = 16$), Ts ($n = 15$), and Ts+ ($n = 13$). One month prior to behavioral testing, mice were moved to a room with a 13:11-h reversed light-dark cycle (lights off at 8:00 a.m., lights on at 9:00 p.m.), and were singly housed to prevent fighting, which can occur when group-housed male mice of this strain are returned to the home cage following behavioral test sessions. Mice were placed on a food restriction regimen to ensure motivation for food rewards during testing. Target weights were calculated at ∼85% of their *ad libitum* weight.

All protocols were approved by the Institutional Animal Care and Use Committee of Cornell University and conform to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Behavioral Testing

Study 1 initiated testing at ∼12 MO, whereas Study 2 began at 6 MO. For simplicity, the mice in Study 1 will be referred to as "aged mice" and in Study 2 as "young mice."

Subjects were tested individually in 1 of 8 PC-controlled operant chambers. The chambers were manufactured in-house, with the body constructed by Ithaca Plastics Inc. (Ithaca, New York, USA) as described previously (Powers et al., 2017). One wall contained a retractable door, controlling access to a dipper (ENV0302M, MED Associates, East Fairfield, Vermont, USA) that dispensed a liquid food reward (Liquefied AIN-76A; Bio-Serv, Frenchtown, New Jersey, USA). The opposite wall contained five nosepoke response ports, each with a green 4-mA LED embedded on the back surface. Mice were pseudorandomly assigned to chambers so that an equal number of mice from each group was tested in each chamber.

Subjects were tested once per day, 6 days per week, and each session lasted 30 min or 70 trials, whichever came first. Mice were weighed and placed into the chambers by experimenters blinded to genotype and diet. After the test session, each subject was returned to the homecage and fed 30 min later. Chambers were thoroughly cleaned after each session using Odormute (R.C. Steele Co, Brockport, New York, USA). Subjects progressed sequentially through a series of training and attention tasks. The total duration of testing spanned a period of 5 months.

A series of training tasks familiarized the subjects with the test chambers and the sequence of responses necessary to complete a trial for the attention tasks (see Powers et al., 2017). Mice then began a five-choice visual discrimination task. On each trial, one of the five nosepoke response port LEDs was illuminated and remained illuminated until the mouse made a nosepoke into one of the ports or until 32 s elapsed. The location of

the visual cue was pseudorandomized across trials so that the number of cue presentations in each port was balanced for each daily session. The subject was rewarded with the liquid food for making a nosepoke into the illuminated (i.e., correct) port. Following an error, or if the animal failed to nosepoke, the dipper door remained closed and a 5-s time-out period was imposed, signaled by the illumination of a 3W house light on the ceiling of the chamber. The interval between trials was 5 s. Each subject remained on this initial visual discrimination task until it reached a criterion of 80% correct for two of three consecutive sessions. Subjects required ∼600–700 trials to reach the criterion, which is consistent with the results of our prior experiments (Moon et al., 2010; Powers et al., 2016, 2017).

Subjects were then tested for eight sessions on Attention Task 1. This task was the same as the initial visual discrimination task, except that the duration of cue illumination was shortened to 1 second. Subjects were subsequently tested for 18 sessions on Attention Task 2, which imposed a variable delay of 0, 2, or 4 s between trial initiation and cue illumination. The pre-cue delays were presented pseudorandomly so that the number of presentations of each combination of pre-cue delay and response port (1–5) was balanced across each session. If a response was made prior to cue onset (i.e., premature), the trial was terminated, and no cue was presented. Finally, subjects were tested for 10 sessions on Attention Task 3, which likewise imposed a variable pre-cue delay of 0, 2, or 4 s, but also had a variable cue duration of either 0.8, 1.0, or 1.4 seconds. Attention Tasks were each initiated on the day following completion of the prior task.

Several types of errors were possible: (i) making a nosepoke into any port prior to cue onset (premature), (ii) responding to a non-illuminated port (inaccurate), and (iii) failing to respond to any port within 32 s of cue onset (omission).

Statistical Analysis

Statistical analyses were performed using the Statistical Analysis System (Version 9.3; SAS Institute, Cary, North Carolina, USA). Data were analyzed using PROC GLM and PROC GLIMMIX. PROC GLIMMIX is a generalized linear mixed models procedure for conducting repeated measures analyses. Fixed factors for all tasks included genotype and maternal diet. Pre-cue delay and stimulus duration were also included for tasks in which these parameters varied. For Attention Task 2, session block (blocks of three daily sessions) was also included so that we could assess the rate of acquisition as the mice learned the new task rule (i.e., a variable delay was imposed between trial onset and cue illumination). Dependent measures included trials to criterion, errors to criterion, percentage of correct responses, percentage of inaccurate responses, percentage of omission errors, and percentage of premature responses. Planned comparisons were made between Ts and Ts+ mice and between 2N and 2N+ mice. The alpha level was set at $p < 0.05$ for all analyses.

RESULTS

There were no significant main effects of genotype or maternal diet on the number of trials to criterion or errors to criterion on the initial five-choice visual discrimination task. Thus, all subjects began Attention Task 1 with a similar mastery of the basic skills and associations required to perform these tasks.

Attention Task 1 (1 s Cue Duration)

Study 1 (Aged Mice)

In Study 1 (aged mice), analysis of percentage correct for Attention Task 1 revealed a significant main effect of genotype [$F_{(1, 56)} = 44.56$, $p < 0.0001$] indicating that Ts65Dn mice performed more poorly than 2N mice. There was no significant main effect of maternal diet, nor a significant genotype by maternal diet interaction (**Figure 1A**). Analysis of error types revealed a significant main effect of genotype on the percentage of omissions [$F_{(1, 56)} = 21.52$, $p < 0.0001$] indicating that the poor performance of the Ts65Dn mice was driven by an increase in these errors (**Table 1**). Note that this was the first task in which the cue duration was relatively brief, and the trisomic mice were impaired in their ability to detect and respond to these brief cues.

Study 2 (Young Mice)

In Study 2 (young mice), analysis of percentage correct for Attention Task 1 revealed a significant main effect of genotype [$F_{(1,53)} = 50.62$, $p < 0.0001$], with Ts65Dn mice again performing more poorly than 2N counterparts. There was also a significant genotype by maternal diet interaction [$F_{(1,53)} = 5.12$, $p < 0.02$]. Planned comparisons revealed that choline-supplemented Ts65Dn mice performed significantly better than their non-supplemented counterparts ($p < 0.01$; **Figure 1B**).

Analysis of error types revealed significant main effects of genotype on the percentage of omissions [$F_{(1,53)} = 24.58$, $p < 0.0001$] and inaccurate responses [$F_{(1,53)} = 20.66$, $p < 0.001$], indicating that trisomic mice committed a higher percentage of both types of errors (**Table 1**). Planned comparisons indicated that improvement in the choline-supplemented Ts65Dn mice for percentage correct was due to fewer inaccurate responses relative to the non-supplemented Ts65Dn mice ($p < 0.01$). Premature responses were not analyzed for this task because there were no pre-cue delays.

Attention Task 2 (0, 2, or 4 s Variable Pre-cue Delay; 1 s Cue Duration)

In both Study 1 and Study 2, pre-cue delay had a significant effect on performance for all groups of mice; percent correct decreased as the duration of the pre-cue delay increased ($p < 0.0001$). The pre-cue delay also had significant effects on each error type (premature, inaccurate, and omission) for all groups in both studies ($p < 0.0001$).

Study 1 (Aged Mice)

In Study 1 (aged mice), the analysis of percentage correct for Attention Task 2 revealed a significant main effect of genotype [$F_{(1, 56)} = 18.03$, $p < 0.0001$] reflecting significant impairment of the Ts65Dn mice relative to 2N mice (**Figure 2A**). This analysis also revealed a significant genotype by block interaction [$F_{(5,434)} = 9.64$, $p < 0.0001$] indicating a difference in learning rate between 2N and Ts65Dn mice (see **Figure 2C**). Planned comparisons revealed that performance was similar across all

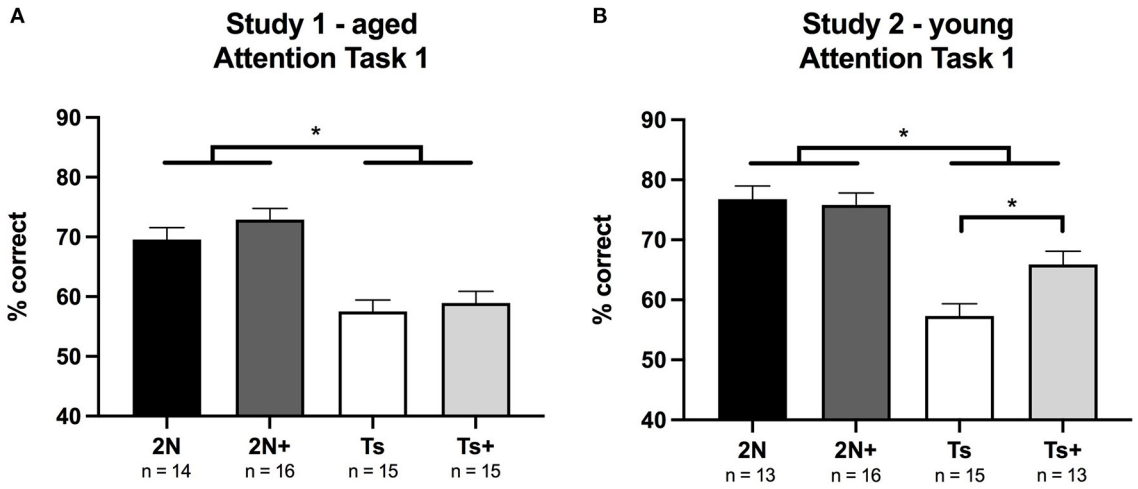

FIGURE 1 | Percentage of correct responses on Attention Task 1 (1 s cue duration) in Study 1 (Panel **A**) and Study 2 (Panel **B**). **(A)** In Study 1 (aged mice), a significant effect of genotype was detected with 2N mice performing better than Ts mice. **(B)** In Study 2 (young mice), a significant effect of genotype was seen as well as a significant genotype by maternal diet interaction. The supplemented trisomic mice performed better than the non-supplemented trisomic mice. $^*p < 0.05$.

TABLE 1 | Average percentage of each error type for each group for each attention task.

		Study 1				Study 2			
		2N	2N+	Ts	Ts+	2N	2N+	Ts	Ts+
Attention Task 1	% inaccurate	18.51 ± 1.91	16.08 ± 1.62	20.49 ± 2.31	18.19 ± 2.11	14.53 ± 1.68	14.24 ± 1.52	18.83 ± 1.71	13.68 ± 1.72
	% omission	6.83 ± 1.24	7.03 ± 1.38	13.81 ± 1.66	15.62 ± 1.73	5.20 ± 1.51	6.82 ± 1.64	11.83 ± 1.57	12.72 ± 1.58
Attention Task 2	% premature	28.33 ± 3.18	26.83 ± 3.21	30.83 ± 3.12	26.33 ± 2.95	27.29 ± 3.24	25.77 ± 2.93	37.48 ± 3.02	28.76 ± 3.25
	% inaccurate	8.48 ± 0.79	6.41 ± 0.82	9.84 ± 0.79	9.82 ± 0.79	6.72 ± 0.91	6.74 ± 0.83	9.51 ± 0.85	8.74 ± 0.92
	% omission	12.05 ± 2.33	13.08 ± 1.89	18.17 ± 1.93	20.67 ± 2.23	8.58 ± 1.61	9.09 ± 1.73	17.31 ± 1.26	14.62 ± 1.78
Attention Task 3	% premature	10.12 ± 1.70	9.21 ± 1.39	13.27 ± 1.38	11.51 ± 1.51	6.63 ± 1.20	8.54 ± 1.08	15.41 ± 1.11	10.20 ± 1.19
	% inaccurate	6.30 ± 1.14	6.26 ± 1.01	7.99 ± 0.96	7.72 ± 1.07	5.19 ± 1.02	5.75 ± 0.93	8.07 ± 0.92	7.97 ± 0.10
	% omission	11.16 ± 2.70	12.26 ± 2.21	20.11 ± 2.19	20.74 ± 2.41	9.28 ± 1.76	8.62 ± 1.59	18.80 ± 1.64	15.38 ± 1.76

four groups during blocks 1 and 2. However, the 2N mice performed significantly better than the Ts65Dn mice during blocks 3–6 ($p < 0.05$). There was no significant effect of maternal diet, nor interactions of diet with either genotype or block. The three-way interaction of genotype, maternal diet, and block was also not significant.

Analysis of the various error types showed significant main effects of genotype on percentage of omissions [$F_{(1, 56)} = 8.58$, $p < 0.01$] and inaccurate responses [$F_{(1, 56)} = 8.97$, $p < 0.01$], but not for premature responses (**Table 1**). There were no significant effects of maternal diet nor an interaction of diet and genotype for any error type.

Study 2 (Young Mice)

In Study 2 (young mice), the analysis of percentage correct for Attention Task 2 revealed a significant main effect of genotype [$F_{(1,53)} = 39.99$, $p < 0.0001$] with Ts65Dn mice performing more poorly than the 2N mice. There was also a significant main effect of maternal diet [$F_{(1,53)} = 4.01$, $p < 0.05$]. Although the genotype by maternal diet interaction was only a trend [$F_{(1,53)} = 2.43$, $p = 0.1$], planned comparisons revealed that within the Ts65Dn mice there was a significant effect of maternal diet ($p < 0.02$) with the Ts+ mice performing better than their non-supplemented counterparts (**Figure 2B**).

A significant genotype by block interaction was seen for percentage correct [$F_{(5,253)} = 15.22$, $p < 0.0001$] as well as a significant maternal diet by block interaction [$F_{(5,253)} = 4.19$, $p < 0.001$]. These effects, illustrated in **Figure 2D**, showed that choline-supplemented Ts65Dn mice performed better than their non-supplemented counterparts during blocks 3–6, although not as well as 2N mice.

Analysis of error types revealed significant main effects of genotype for percentage of omissions [$F_{(1,53)} = 16.84$, $p < 0.001$], percentage of inaccurate responses [$F_{(1,53)} = 9.75$, $p < 0.01$], and percentage of premature responses [$F_{(1,53)} = 4.49$, $p < 0.05$] (**Table 1**). There were no significant main effects

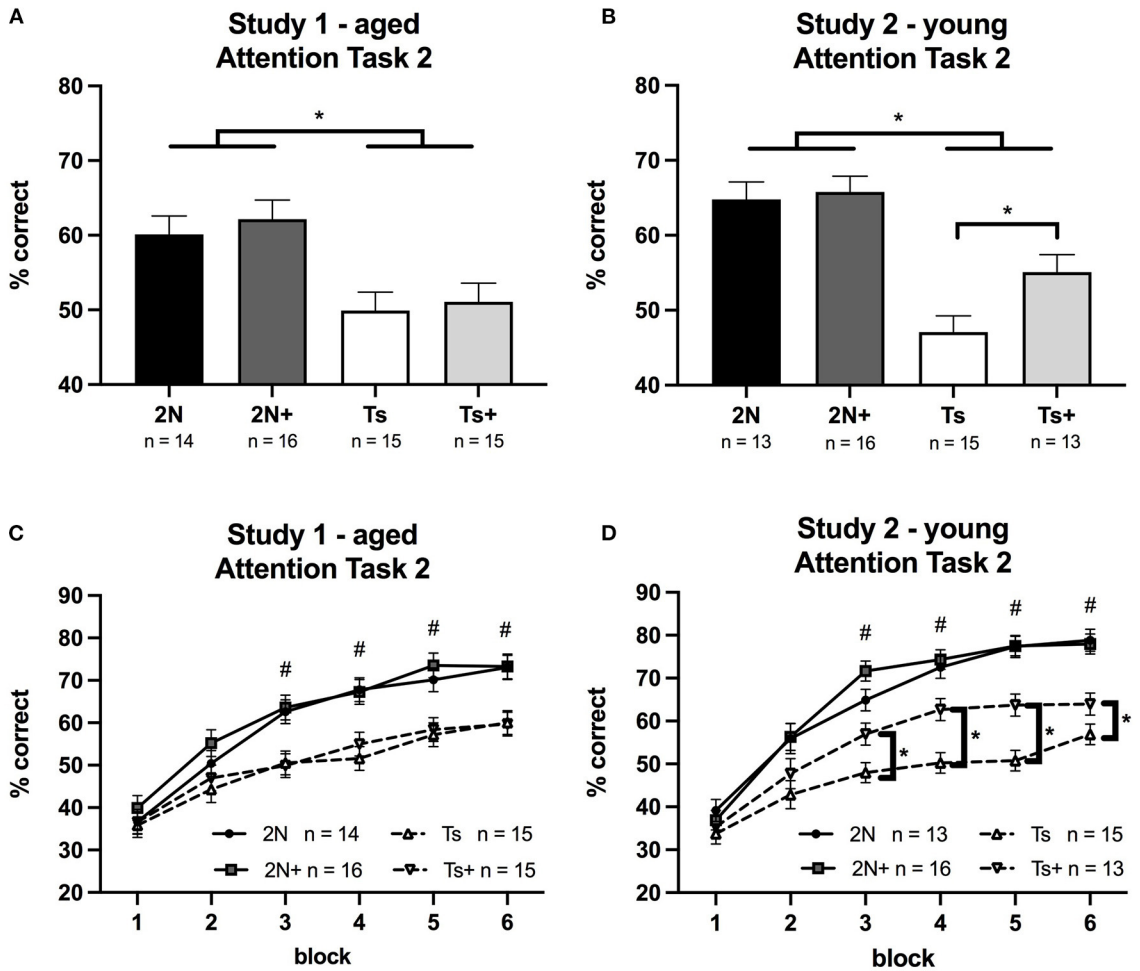

FIGURE 2 | Percentage of correct responses on Attention Task 2 (1 s cue duration; 0, 2, or 4 s pre-cue delay) in Study 1 and Study 2. Panels **(A,B)** depict percent correct averaged across the entire task, whereas Panels **(C,D)** depict performance as a function of testing block. **(A)** In Study 1 (aged mice), a significant effect of genotype was detected, reflecting the superiority of the 2N vs. trisomic mice. **(B)** In Study 2 (young mice), a significant effect of genotype was detected as well as a significant effect of maternal diet. Planned comparisons revealed that within the Ts65Dn mice, there was a significant effect of maternal diet ($p < 0.02$) with the supplemented trisomic mice performing better than non-supplemented trisomic mice. **(C)** In Study 1 (aged mice), there was a significant genotype by block interaction with 2N mice performing better than Ts mice during blocks 3–6. **(D)** In Study 2 (young mice), there was a significant genotype by block interaction and a significant maternal diet by block interaction. #The 2N and 2N+ groups were both significantly different from each Ts group ($p < 0.05$). *The supplemented trisomic mice performed better than the non-supplemented trisomic mice during blocks 3–6 ($p < 0.05$).

of maternal diet, nor any significant genotype by maternal diet interactions. For premature response errors, there were significant genotype by block [$F_{(5,140)} = 6.81$, $p < 0.0001$] and maternal diet by block [$F_{(5,140)} = 2.34$, $p < 0.05$] interactions. Planned comparisons indicated that the non-supplemented Ts65Dn mice made significantly more premature responses than the supplemented trisomic mice ($p < 0.05$).

Attention Task 3 (0, 2, or 4 s Variable Pre-cue Delay; 0.8, 1, or 1.4 s Variable Cue Duration)

In both Study 1 and Study 2, pre-cue delay had a significant effect on performance for all groups of mice; percentage correct decreased as the duration of the pre-cue delay increased ($p < 0.0001$). Likewise, cue duration had a significant effect on performance for all subjects; percentage correct decreased as the cue duration decreased ($p < 0.0001$). Pre-cue delay also had significant effects on each error type (premature, inaccurate, omission) for all groups in both studies ($p < 0.0001$). Cue duration had significant effects on inaccurate responses and omissions for all groups in each study ($p < 0.0001$).

Study 1 (Aged Mice)

In Study 1 (aged mice), analysis of percentage correct for Attention Task 3 revealed a significant main effect of genotype [$F_{(1, 56)} = 19.41$, $p < 0.0001$] with Ts65Dn mice performing significantly worse than the 2N mice (**Figure 3A**). There was no significant effect of maternal diet nor was there a significant

genotype by maternal diet interaction. Analysis of errors showed a significant main effect of genotype on the percentage of omissions [$F_{(1, 56)} = 10.28$, $p < 0.01$] (**Table 1**). There were no significant effects of maternal diet on any error type.

Study 2 (Young Mice)

In Study 2 (young mice), the analysis of percentage correct for Attention Task 3 revealed a significant main effect of genotype [$F_{(1, 53)} = 50.31$, $p < 0.0001$] reflecting the fact that the 2N mice outperformed the Ts65Dn mice. In addition, there was a significant genotype by maternal diet interaction [$F_{(1, 53)} = 4.67$, $p < 0.05$]. The choline-supplemented Ts65Dn mice performed significantly better than their non-supplemented counterparts ($p < 0.01$; **Figure 3B**). The analysis also revealed a significant genotype by maternal diet by delay interaction for percentage correct [$F_{(2, 100)} = 3.16$, $p = 0.04$]. While we observed no effect of MCS in the aged mice (**Figure 3C**), there was a significant effect of maternal diet in the young Ts65Dn mice (**Figure 3D**) on trials with a 0 s pre-cue delay ($p < 0.01$) and on trials with a 4 s pre-cue delay ($p < 0.01$), where choline-supplemented Ts65Dn mice performed better than their non-supplemented counterparts. A similar pattern was seen for trials with a 2 s pre-cue delay, but the contrast was not statistically significant.

Analysis of error types indicated significant main effects of genotype on percentage of omissions [$F_{(1, 53)} = 24.72$, $p < 0.0001$], inaccurate responses [$F_{(1, 53)} = 8.46$, $p < 0.01$], and premature responses [$F_{(1, 53)} = 20.62$, $p < 0.0001$] (**Table 1**); in all cases the trisomic mice made more errors than the 2N. In addition, there was a significant genotype by maternal diet interaction [$F_{(1,53)} = 9.92$, $p < 0.01$] for percentage of premature responses. The non-supplemented Ts65Dn mice made significantly more premature responses than Ts+ mice ($p < 0.01$).

DISCUSSION

We reported on the impact of MCS in the Ts65Dn mouse model of DS, assessing learning and attention in two cohorts of mice; one cohort tested at 12 MO (Study 1), and a second at 6 MO (Study 2). In the younger cohort, Ts65Dn offspring born to dams consuming a choline-supplemented diet performed significantly better than their non-supplemented counterparts on each of the three increasingly challenging attention tasks. By contrast, this cognitive benefit of MCS was not observed in the older cohort of Ts65Dn subjects. These findings from young mice replicate and extend the results of our prior study in which testing began at 6 MO (Moon et al., 2010). This earlier study also demonstrated substantial cognitive benefits of MCS for the trisomic offspring. In contrast, the results from the aged mice are similar to our earlier study in which behavioral testing began at 12 MO (Powers et al., 2017). These prior results showed impairment in Ts65Dn mice, but only minor cognitive improvement in choline-supplemented Ts65Dn offspring. Collectively, these four studies indicate that MCS has long-lasting benefits on learning and attention, but the positive effects diminish with age, possibly due to the progression of AD-related neuropathology.

Nature of the Ts65Dn Impairment and the Benefit Provided by MCS

Important information about the nature of the impairment of the trisomic mice was provided by the types of errors that differentiated them from 2N mice, and also by the pattern of group differences across the different tasks, each with unique cognitive demands. In Task 1, the visual cue was presented at trial onset (as in the prior visual discrimination training task), but the duration of the cue was much shorter than for the training task, which proved to be especially challenging for the Ts65Dn mice. All groups had attained a baseline of at least 80% correct on the training task. However, performance of the 2N mice only modestly declined with the briefer cue. In contrast, the performance of the Ts65Dn mice dropped considerably (**Figure 1**), primarily due to increased omission errors. In a prior study, videotape analysis revealed that the increased omission errors of the trisomic mice are due to being off-task (facing the side of the chamber opposite to the response ports) (Driscoll et al., 2004). This behavior may be due to cognitive decline in Ts65Dn mice, as they begin to show age-related loss of BFCNs and marked septohippocampal deficits by 6 MO (Granholm et al., 2000; Kelley et al., 2014, 2016).

Task 2 presented the animals with a new rule, which uncovered additional impairments in the trisomic mice. In this task, the visual cue was presented after a variable delay. A response prior to cue onset terminated the trial and was tallied as a premature response error. Since this a difficult task to learn, it initially results in many errors and a dramatic decline in reinforcement rate (relative to the prior task). Previous analysis of videotapes of the animals performing this task revealed that Ts65Dn mice exhibit repetitive jumping specifically after committing an error, indicative of impaired affect regulation; 2N mice do not show this behavior (Driscoll et al., 2004; Moon et al., 2010). Thus, the rate of learning this task reflects emotion regulation, as well as inhibitory control and attentional control. As seen in **Figure 2**, all groups exhibited poor performance during the first two session blocks (six sessions) on this task due to a high rate of premature responses. By the third session block, the 2N mice showed clear signs of having learned the new rule, performing significantly better than the trisomic mice. This genotype effect was observed in both young and aged mice. This genotype difference in performance during the learning process was due to a higher rate of premature responses, inaccurate responses, and omissions by the Ts65Dn mice reflecting deficiencies in inhibitory control, associative ability, and emotion regulation (Driscoll et al., 2004; Moon et al., 2010; Powers et al., 2017).

Task 3 increases demand on attention and inhibitory control because, in addition to the variability of the pre-cue delay, the cue duration is also variable and very brief on some trials. Ts65Dn mice continued to make more omission-, inaccurate-, and premature response errors than the 2N controls. The most predominant error type in this task in the aged Ts65Dn mice was omissions. However, the trisomic mice also committed significantly more premature and inaccurate responses than the 2N mice, indicative of impaired inhibitory control and impaired attention, respectively.

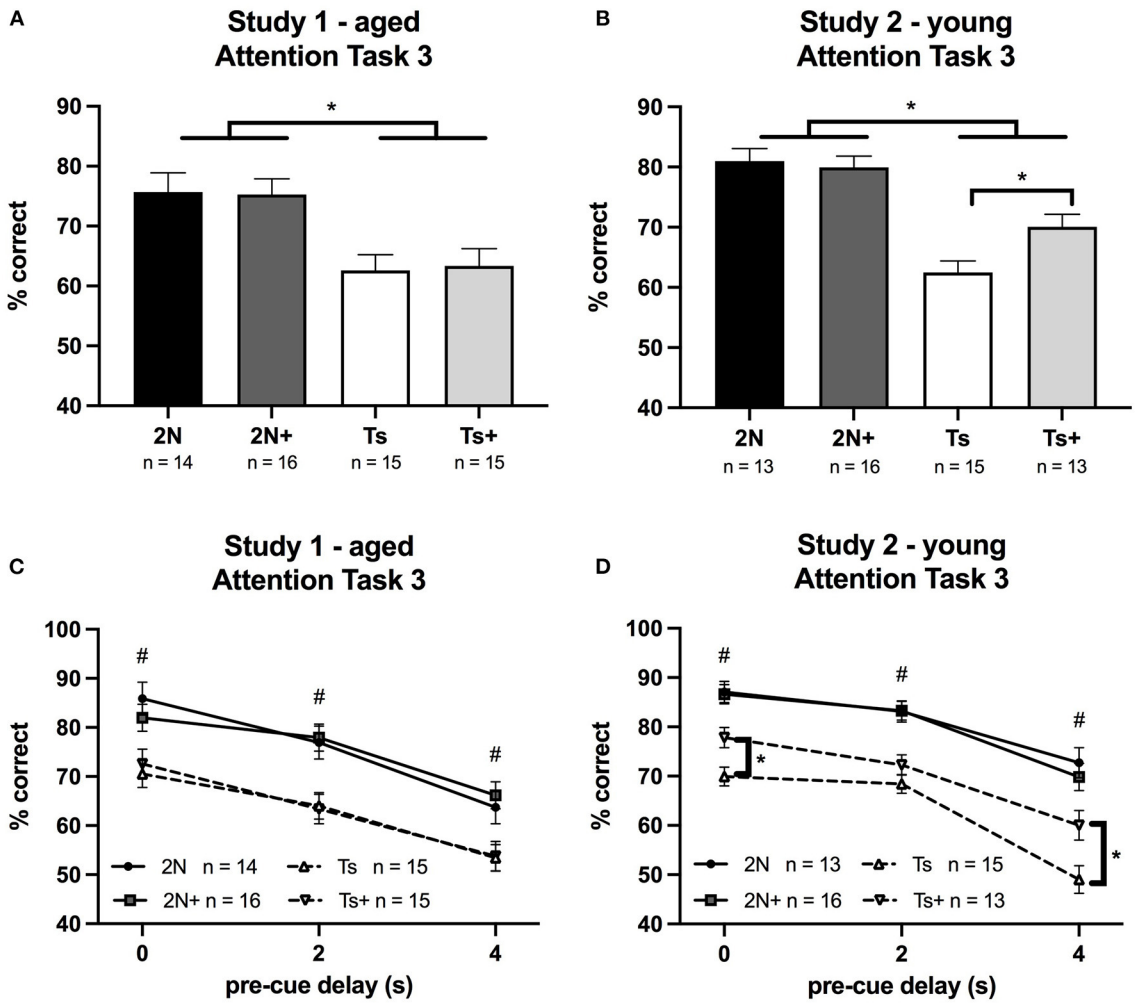

FIGURE 3 | Percentage of correct responses on Attention Task 3 (0.8, 1, or 1.4 s cue duration; 0, 2, or 4 s pre-cue delay) in Study 1 and Study 2. Panels **(A,B)** depict percent correct averaged across the entire task, whereas Panels **(C,D)** depict performance as a function of pre-cue delay. **(A)** In Study 1 (aged mice), a significant effect of genotype was seen, with 2N mice performing better than Ts mice. **(B)** In Study 2 (young mice), a significant effect of genotype was detected as well as a significant genotype by maternal diet interaction. The supplemented trisomic mice performed better than the non-supplemented trisomic mice. **(C)** In Study 1 (aged mice), a significant effect of genotype was detected. **(D)** In Study 2 (young mice), there was a significant genotype by maternal diet by delay interaction. #The 2N and 2N+ groups were both significantly different from each Ts group ($p < 0.05$). *The supplemented trisomic mice performed significantly better than the non-supplemented trisomic mice on trials with a 0 s delay and trials with a 4 s delay ($p < 0.05$).

Several aspects of dysfunction in Ts65Dn mice were ameliorated by increased maternal choline intake. Insight into the nature of the MCS benefit was provided by inspection of the types of errors that exhibited improvement, and also a consideration of the unique demands of each task. In Task 1, the young supplemented trisomic mice displayed improved performance relative to the non-supplemented trisomic mice (**Figure 1B**) due to a reduction in inaccurate responses. Thus, the benefit of MCS observed in trisomic mice (vs. non-supplemented) on this task likely reflects an improved ability to detect and respond to the now briefer cues (i.e., improved attentional function). On Task 2, the young Ts+ mice were markedly improved relative to their non-supplemented counterparts due to a reduction in premature response errors. When considering task demands, this MCS benefit likely reflects improved inhibitory control, associative ability, and emotional regulation, supporting our earlier findings (Moon et al., 2010). On Task 3, the young Ts+ mice likewise demonstrated improved performance relative to non-supplemented Ts65Dn mice due to a reduction in premature responses indicative of improved inhibitory control in the supplemented mice.

Omission errors contributed to the impaired performance of the Ts65Dn mice in all three tasks, but this error type was unaffected by MCS. Again, based on our videotape analysis of aged Ts65Dn mice (Driscoll et al., 2004), this type of error is associated with the mice being off-task, and not attending to the visual cues, and may reflect cognitive decline associated with AD-like pathological changes. Omission errors were especially

predominant in the aged Ts65Dn mice, which did not show a benefit from MCS on these attention tasks. Although the young Ts+ mice demonstrated improved performance (relative to their non-supplemented counterparts), due to reductions in inaccurate and premature response errors, the aged Ts+ mice showed no benefit from MCS. Perhaps, the progression of age-related cholinergic neuropathology overrides the neurological benefits of MCS seen during the younger ages.

In our two prior studies of MCS, we found significant benefits of the added choline for the 2N mice (in addition to the trisomics) although the benefits were only seen at very specific points in the testing, and the effects were small in magnitude (Moon et al., 2010; Powers et al., 2017). In this study, a comparison of the 2N and 2N+ mice revealed trends toward improved performance in the 2N+ mice at identical points in the testing, but the effects were not statistically significant. Specifically, in our previous study of aged mice (Powers et al., 2017), we observed a significant main effect of maternal diet on Attention Task 1, and in fact, a similar trend was seen in this study of aged mice (**Figure 1A**). In our previous study of young mice, benefits of the MCS were seen only in Attention Task 2 when the animals were first introduced to pre-cue delays: the 2N+ mice were superior to their 2N counterparts during the early learning phase. In this study of young mice, at this identical point in the learning process of Attention Task 2 (block 3), the 2N+ mice tended to perform better than their 2N counterparts (see **Figure 2D**), but failed to achieve statistical significance. It appears that the benefits of MCS in the 2N mice are subtle, and may not always achieve significance in relatively small samples. In addition, it should be considered that a ceiling effect contributed to the lack of differences between the 2N and 2N+ groups since both the groups performed successfully (∼80% correct) on even the most challenging tasks. Prior reviews have noted that the cognitive benefits of MCS for normal rodents are evidenced only under demanding testing conditions (Meck and Williams, 2003; McCann et al., 2006). It was not possible to further increase task difficulty in this study due to the impairment of the trisomic mice.

Conclusions

The findings of this study suggest that supplementing the maternal diet with extra choline during pregnancy is an extremely promising, high-benefit, low-risk intervention to reduce the cognitive and affective dysfunction in DS. Our translational research using a mouse model of DS has demonstrated that increasing maternal intake of choline during pregnancy and lactation significantly improves attentional function, spatial cognition, and affect regulation in trisomic offspring. These supplemented offspring also exhibit protection of medial septal cholinergic neurons, as well as normalization of hippocampal neurogenesis and expression of genes associated with synaptic plasticity, calcium signaling, and neurodegeneration (Velazquez et al., 2013; Ash et al., 2014; Alldred et al., 2018, 2019). Indeed, there is growing evidence that all pregnant women should increase their intake of choline. Pregnant women in the USA consume on average only ∼70% of the Choline Adequate Intake (AI) level recommended by the National Academy of Medicine [Institute of Medicine (US) Standing Committee on the Scientific Evaluation of Dietary Reference Intakes its Panel on Folate, 1998], which is of significant concern in light of the crucial roles that choline plays in fetal neurodevelopment. Moreover, the functional benefit of increasing maternal choline intake, for all pregnancies, is indicated by the results of recent studies that experimentally manipulated choline intake during pregnancy and measured child cognitive outcomes. These studies revealed that higher choline intakes are not only safe and well-tolerated by the mothers, but also improve indices of child cognitive functioning, assessed during infancy (Caudill et al., 2018), toddlerhood (Ross et al., 2016), and 7 years of age (Bahnfleth et al., 2021). There is growing pressure to change obstetric policy so that choline is included in standard prenatal vitamin regimens, which is not currently the case (Caudill et al., 2020). Based on existing animal and human studies, addition of choline to a prenatal vitamin regimen would have population wide benefits likely resulting in improved cognition and affect regulation in all children, as well as providing an early intervention for DS that may protect against AD-associated cognitive decline.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of Cornell University.

AUTHOR CONTRIBUTIONS

BEP conducted all experiments, performed analytic calculations with support from MSS and BJS, and wrote the manuscript with support from BJS and RV. BJS, SDG, and EJM conceived and planned experiments. All authors provided critical feedback to help shape the research, analysis, and manuscript.

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