INTELLECTUAL DISABILITIES IN DOWN SYNDROME FROM BIRTH AND THROUGHOUT LIFE: ASSESSMENT AND TREATMENT

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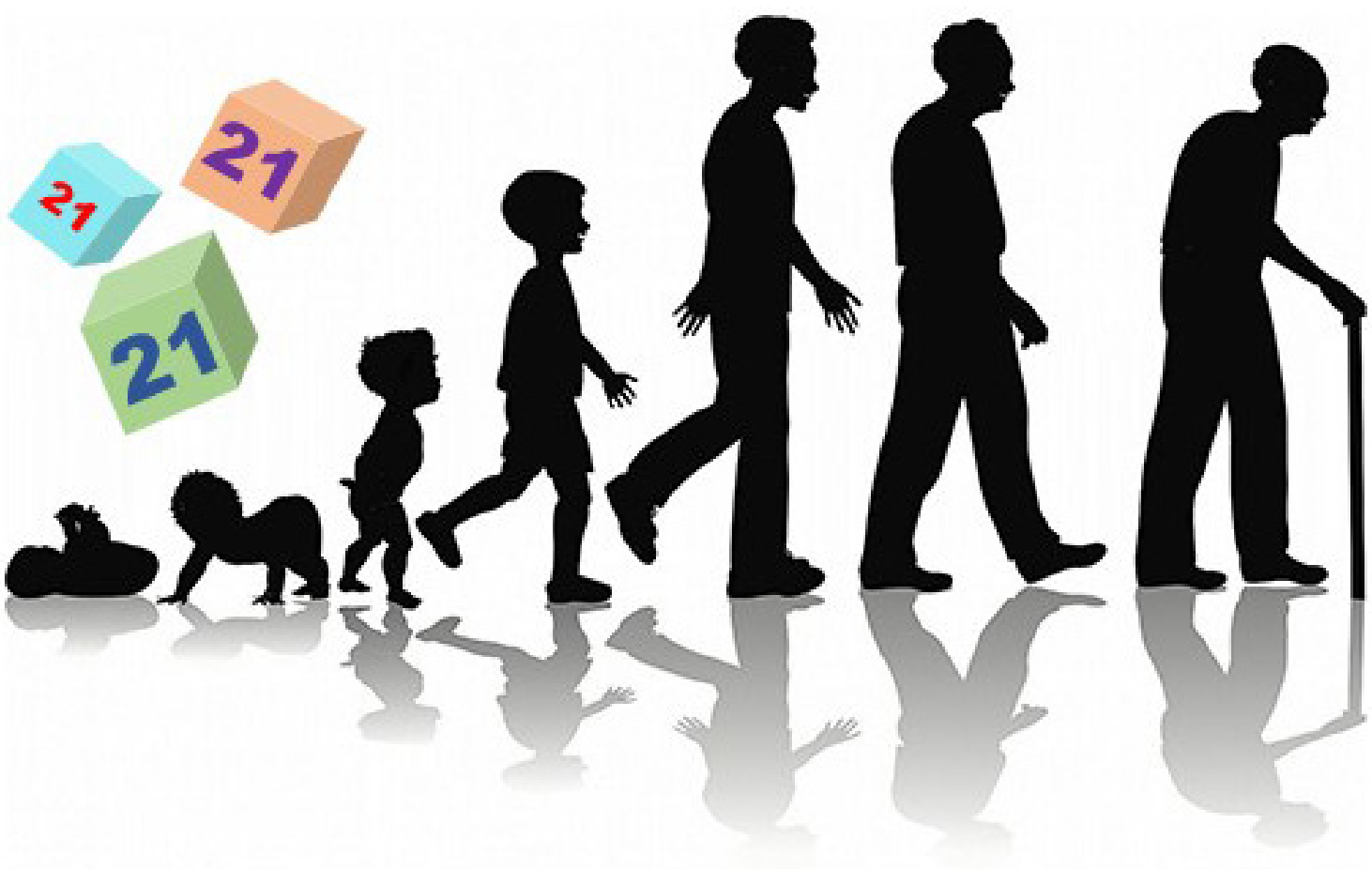

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INTELLECTUAL DISABILITIES IN DOWN SYNDROME FROM BIRTH AND THROUGHOUT LIFE: ASSESSMENT AND TREATMENT

Topic Editors:
Marie-Claude Potier, Sorbonne Universités, France
Roger H. Reeves, Johns Hopkins University School of Medicine, USA

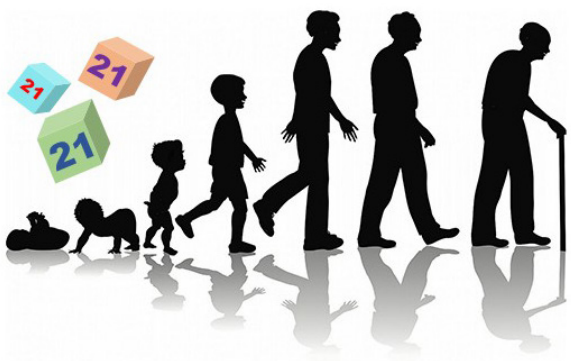

Figure by Marie-Claude Potier

Research on the multiple aspects of cognitive impairment in Down syndrome (DS), from genes to behavior to treatment, has made tremendous progress in the last decade. The study of congenital intellectual disabilities such as DS is challenging since they originate from the earliest stages of development and both the acquisition of cognitive skills and neurodegenerative pathologies are cumulative. Comorbidities such as cardiac malformations, sleep apnea, diabetes and dementia are frequent in the DS population, as well, and their increased risk provides a means of assessing early stages of these pathologies that is relevant to the general population. Notably, persons with DS will develop the histopathology of Alzheimer's disease (formation of neuritic plaques and tangles) and are at high risk for dementia, something that cannot be predicted in the population at large. Identification of the gene encoding the amyloid precursor protein, its localization to chromosome 21 in the 90's and realization that all persons with DS develop pathology identified this as an important piece of the amyloid cascade hypothesis in Alzheimer's disease. Awareness of the potential role of people with DS in understanding progression and treatment as well as identification of genetic risk factors and also protective factors for AD is reawakening.

For the first time since DS was recognized, major pharmaceutical companies have entered the search for ameliorative treatments, and phase II clinical trials to improve learning and memory are in progress. Enriched environment, brain stimulation and alternative therapies are being tested while clinical assessment is improving, thus increasing the chances of success for therapeutic interventions. Researchers and clinicians are actively pursuing the possibility of prenatal treatments for many conditions, an area with a huge potential impact for developmental disorders such as DS.

Our goal here is to present an overview of recent advances with an emphasis on behavioral and cognitive deficits and how these issues change through life in DS. The relevance of comorbidities to the end phenotypes described and relevance of pharmacological targets and possible treatments will be considerations throughout.

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EDITORIAL
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Editorial: Intellectual Disabilities in Down Syndrome from Birth and Throughout Life: Assessment and Treatment

Marie-Claude Potier[1]* *and Roger H. Reeves*[2]*

[1] Centre National de la Recherche Scientifique UMR 7225, Institut National de la Santé et de la Recherche Médicale, U1127, Institut du Cerveau et de la Moelle, Sorbonne Universités, UPMC Univ Paris 06 UMR S 1127, Paris, France, [2] Department of Physiology and McKusick Nathans Institute for Genetic Medicine, Johns Hopkins University School of Medicine, Baltimore, MD, USA

Keywords: down syndrome, intellectual disabilities, Alzheimer's disease, treatment, prenatal, language, GABA

The Editorial on the Research Topic

Intellectual Disabilities in Down Syndrome from Birth and Throughout Life: Assessment and Treatment

Research on the multiple aspects of cognitive impairment in Down syndrome (DS), from genes to behavior to treatment, has made tremendous progress in the last decade as reflected in current clinical trials to improve learning and memory. Congenital intellectual disabilities such as DS originate from the earliest stages of development and both the acquisition of cognitive skills and neurodegenerative pathologies are cumulative. Comorbidities such as cardiac malformations, sleep apnea, diabetes, and dementia are frequent in the DS population, as well, and their increased risk in this genetically sensitized population provides a means of assessing early stages of these pathologies that affect the entire population.

Persons with DS will develop the histopathology of Alzheimer's disease (neuritic plaques and tangles) due to over-expression of genes on chromosome 21, notably the amyloid precursor protein. Thus, the DS population is at high risk for dementia, something that cannot be predicted in the population at large. Awareness of the potential role of people with DS in understanding progression and treatment as well as protective factors for AD is reawakening.

Major pharmaceutical companies have entered the search for ameliorative treatments for features of DS, and phase II clinical trials to improve learning and memory are in progress. Enriched environment, brain stimulation, and alternative therapies are being tested while clinical assessment is improving, thus increasing the chances of success for therapeutic interventions. Researchers and clinicians are actively pursuing the possibility of prenatal treatments for many conditions, an area with a huge potential impact for developmental disorders such as DS but which also faces significant challenges to assure safety and to assess outcomes. One major barrier to these studies is that there is no current way to predict the severity of cognitive (or most other) effects in DS, and thus it is not possible to determine whether an intervention has had a positive effect. This problem is exacerbated because evaluation of the cognitive state of young babies is at an early stage.

Our goal here is to present an overview of recent advances with an emphasis on behavioral and cognitive deficits and how these issues change through life in DS. The relevance of comorbidities to the end phenotypes described and relevance of pharmacological targets and possible treatments will be considerations throughout. This Topic contains seven original research articles, five reviews, and one perspective article.

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Edited and reviewed by:
Nuno Sousa,
University of Minho, Portugal

***Correspondence:**
Marie-Claude Potier
marie-claude.potier@upmc.fr;
Roger H. Reeves
rreeves@jhmi.edu

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Eight papers are related to clinical work in individuals with DS.

Liogier d'Arduy et al. from Hoffmann La Roche laboratory, in their original research article, publish for the first time a redefined cognitive scale to assess executive function, memory, and language in DS individuals from 12 to 30 years. They developed a multicenter observational, non-pharmacological, and longitudinal study based on a 90 min testing period with one or two breaks over 6 months with three visits starting at birth and extending 1–6 months. This study draws the list of tests that are currently used for the ongoing 26-week Phase 2 study of Basmisanil in young individuals with DS which was recently extended from 12 to 30 years down to age 6 for IQ measurement, memory testing using list learning, executive function, and language assessments that are test–retest reliable and have no floor effect.

In their review, Edgin et al. propose to consider the end-state of DS cognitive phenotypes emergent across developmental time. For language, which is more impaired in children with DS than expected considering their general mental age, intervention targeting the very early neural roots of language will be important. Brain imaging data indicate that network connectivity is more diffuse in adults with DS with increased local network synchrony and under-connectivity of long-range connections leading to language impairments. Sleep disturbances are present in most children and adults with DS and could be very detrimental for hippocampal memory consolidation and word learning, having cascading implications for language comprehension and everyday social interactions.

Two original research articles are related to language problems in individuals with DS. De Hoyo et al. study semantic verbal fluency pattern in young non-demented adults with DS. They found a clear deficit in retrieval of words (lexicon) beyond the accession of common words. These values are correlated with Aβ42 plasma levels. The semantic verbal fluency test may be useful to predict risk of dementia in individuals with DS. Channell et al. compare narrative language performances of children with DS and Fragile X-syndrome (FXS). They used an episode-based coding scheme to examine macrostructures and microstructures from stories produced in response to a wordless picture book. Individuals with DS acquired the conceptual knowledge for expressing the key story elements but their narrative macrostructure was impaired, they showed limited expressive syntactic abilities and had difficulties talking about others' perspectives and intentions. These deficits are shared with FXS. Children with DS take more time to tell a story and use less verbs than those with FXS.

In their research article, Lee et al. study executive function profiles in DS. Children with DS have deficits in "cool" executive functions such as working memory and planning and fewer deficits in "hit" executive functions involving behavioral inhibition and emotional control (Lee et al.). These deficits are relatively stable across development until young adulthood. Higher-level cognition abilities will have to be evaluated later in life.

Mc Guire and Defrin review acute and chronic pain experienced in people with DS, an area where research is limited. Acute pain appears to be delayed and once perceived it gets magnified and persists for a longer period of time. Studies remain to be done on information processing in DS including cognitive appraisals of the pain, emotional, and behavioral response, and social context. DS poses an increased risk to experience pain due to congenital abnormalities and environmental risk factors, and this can be exacerbated when affected individuals have difficulties expressing their pain.

Rafii et al. report the feasibility study of the DSBI (Down Syndrome Biomarker Initiative) on non-demented individuals with DS. They found greater hippocampal atrophy with amyloid load and an inverse correlation of amyloid load with regional glucose metabolism. Interestingly they could identify amyloid plaques in the retina. This pilot study shows that biomarkers of AD can be used in DS to assess AD pathology and will be useful for characterizing larger cohorts and defining readouts for future clinical trials of disease modifiers.

Finally, Nizetic et al. discuss the dual role of APP in DS and AD. Familial cases of AD with microduplication of the APP gene have peculiar pathology with prominent amyloid angiopathy but do not show intellectual disabilities while individuals with DS rarely show vascular and mixed dementia but intellectual disabilities are prevalent. Beyond AD, APP, and Aβ could potentially affect cognitive dysfunction in DS. The balance between beneficial and deleterious effects of neuronal activity in DS is still an open question that will need to be answered in order to design optimal treatments.

The remaining manuscripts deal with pharmacotherapy and mouse models for DS and AD in DS.

Souchet et al. present new set of data suggesting the important role of Dyrk1A in the control of excitation/inhibition imbalance in DS. They identify changes of expression for a set of proteins involved in excitation or inhibition and further show that green tea extracts containing EGCG can restore levels of most of these markers in adult mice overexpressing Dyrk1a alone or in Ts65Dn mice. Some of the reported effects of EGCG are likely due to the presence of caffeine in various extracts, however, decaffeinated extracts still have a beneficial effect both on behavioral deficits and on brain markers.

Catuara-Solarz et al. show that a combination of EGCG and enriched environment in 5–6 month old Ts65Dn mice rescued hippocampal-dependent learning and memory while either alone did not. In their study, they developed a new statistical analysis that identifies a large degree of variance caused by memory-unrelated effects that could be applied to better integrate interindividual variations.

Duchon and Hérault review the crucial role of Dyrk1A in intellectual disability in Autosomal Dominant Mental Retardation 7 (MRD7). They review potential Dyrk1A inhibitors such as harmine, flavonoids, catechine, and other natural products or synthetic compounds which all target the ATP binding site but also affect other kinases.

Stagni et al. review 34 studies of potential prenatal therapies that have been tested in Ts65Dn mice, providing preclinical data that could be applied to perinatal treatment of DS. Fetuses with DS have brain defects altering neuronal network formation and functioning. They report only three perinatal treatments (Shh

agonist, fluoxetine, and EGCG) while five were administered prenatally (choline, fluoxetine, three treatments against oxidative stress, and EGCG). The authors suggest that treatment of fetuses with DS during weeks 12–16 of human pregnancy may have a significant impact on neurogenesis. Pilot studies are proposed with fluoxetine, although outcome measures remain unclear (see below).

Choong et al. review the literature on mouse models of Alzheimer's pathology and dementia in DS. They nicely present data on people with DS and familial AD with either APP mutations or APP microduplication and discuss the clinical assessment of dementia in DS individuals who have baseline cognitive impairments. They further discuss the involvement of genes from Hsa21 in AD pathology, highlighting the need for studying mouse models of AD-DS, and extending biomarker studies that are being undertaken in large cohorts of people with DS thus contributing to the elucidation of genotype–phenotype relationships that ultimately lead to dementia.

We have selected contributions for this volume to touch on the state of progress in a number of immediate areas for translation. Of course, as one of the most complex genetic challenges compatible with human survival past term, trisomy 21 remains a formidable challenge for translational studies.

From a basic science standpoint, additional animal models would be useful for DS and for AD and the relationship between them. The mouse has proven to be pre-eminent for genetic studies, but existing behavior paradigms for mice need to be expanded for aging studies. Further, mice only develop a subset of the histopathology associated with DS and AD, and then only when engineered to contain mutations that are strongly predisposing. Larger models allowing more refined behavior analysis, better access to anatomical structures and an additional perspective of how to understand the relevance of animal pathology to human conditions would be highly valuable. With the advent of CRISPR-Cas9 technology, it may be possible to develop models of DS and of DS-AD in the rat and in small primates (e.g., marmoset).

In the translational interface, the current trials of Basmisanil (CLEMATIS NCT02024789 in adult and adolescents with DS and NCT02484703 in children 6–11 years with DS) and BTD-001 (Balance Therapeutics, ACTRN12612000652875 on the Australian—New Zealand clinical trial registry) are based substantially on findings in the Ts65Dn mouse model, established from extensive behavioral, electrophysiological, and biochemical assessments. A rather large number of different drugs/supplements/exercise therapies have been demonstrated to improve performance in learning and memory assays in these mice (as reviewed by Stagni et al.), and some treatments have been assessed for impact on neurogenesis or neuroanatomy, as well. While the findings regarding GABAergic transmission in Ts65Dn provided a powerful incentive to move treatments toward the clinic, substantive support for likely mechanisms is highly desirable. In particular, it would be extremely useful to explain why treatments with a large variety of molecules selective for different pharmacological targets can all provide a similarly beneficial behavioral impact in Ts65Dn. Another consideration is the pharmaco-chemistry behind treatments, especially those involving food extracts such as green tea extracts containing EGCG. Commercially available supplements are complex mixes of compounds well-documented to vary in composition and concentration. In many cases, half-lives and toxicity are not precisely described. Thorough assessment of purified target compounds, coupled with pharmacokinetics of how they are metabolized or the synthesis of pure analogs will be an important next step in moving these compounds to the clinic.

A critical next step for DS and for AD is the development of biomarkers, especially for early (pre-) stages of disease. The DS population can be immensely informative in this regard since all individuals with trisomy 21 develop the histopathology of AD, while a subset develop dementia by age 60 despite decades of exposure to elevated amyloid in various forms. Extension of findings in this area to fluid biomarkers—so-called "liquid biopsy"—would be tremendously useful. Discovery of biomarkers that could predict high risk for dementia would be very useful before applying neuroprotective or anti-amyloid treatments, such as the ones described recently by Dekker et al. (2015). These studies may also indicate metabolic or biochemical differences reflecting molecules that are protective against disease progression. Correlating these with increasingly informative brain imaging approaches may be of use to the entire population, not just those with DS.

CONCLUSIONS

Clinical applications demand improved testing for and better understanding of cognitive development and its impairments in DS throughout life. Learning and memory experts are defining specific aspects of cognition that are affected in DS and these tests are being validated at ever earlier ages. These developments will be critical to a clearer understanding of both ends of life in DS. At present, the race to perinatal treatment appears to us to lack critical elements, most notably any possible outcome measures short of "normality." We would caution that such an elusive goal cannot be supported only by a few behavior tests in a distantly related species. While the ability exists to screen for some structural anomalies prenatally (e.g., heart defects) there is currently no method to predict occurrence or severity of impact on learning and memory, the likelihood of autistic behaviors, or other cognitive outcomes in a given individual with DS. At a minimum, development of predictive biomarkers needs to be studied in longitudinal assessments before fetuses and babies are exposed to drugs that have the potential to do harm at critical periods, especially in untested combinations. In the risk-benefit equation, absence of any quantifiable, reproducible outcome prediction means there is zero gain, therefore risk is hardly acceptable. The potential impact of prenatal treatments for a disorder that arises substantially due to perturbations in development is obvious and this should make development of a natural history of DS that includes biomarkers, clinical endpoints, and repeatable, validated behavior testing a priority of the highest order for DS research. The appropriate ages and duration for these treatments remain to be clarified and long term effects will need to be elucidated.

In the necessarily narrow sampling of recent activities in the DS research community presented here, we have tried to highlight current developments across areas that relate to cognitive therapy in DS. These discoveries span the entire life, from pre-natal development to age-related pathologies. It is rather shocking to note that although trisomy 21 is the most common genetic cause of intellectual disability whose proximate cause has been known for more than 50 years and the existence of which syndrome has been recognized for more than 150 years, very little is known about the natural history of DS or even of co-morbidities of penetrance or expressivity among the multiple possible outcomes. We can and must do better for these members of society and recognize that knowledge gained from those with a genetic predisposition with a number of possible deleterious outcomes is applicable to all.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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Everyday executive functions in Down syndrome from early childhood to young adulthood: evidence for both unique and shared characteristics compared to youth with sex chromosome trisomy (XXX and XXY)

Nancy Raitano Lee[1,2], Payal Anand[3], Elizabeth Will[4], Elizabeth I. Adeyemi[1], Liv S. Clasen[1], Jonathan D. Blumenthal[1], Jay N. Giedd[1,5], Lisa A. Daunhauer[4], Deborah J. Fidler[4] and Jamie O. Edgin[3]*

[1] Child Psychiatry Branch, National Institute of Mental Health, Bethesda, MD, USA, [2] Department of Psychology, Drexel University, Philadelphia, PA, USA, [3] Department of Psychology, University of Arizona, Tucson, AZ, USA, [4] Human Development and Family Studies, Colorado State University, Fort Collins, CO, USA, [5] Department of Psychiatry, University of California, San Diego, La Jolla, CA, USA

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Edited by:
Marie-Claude Potier, Centre National de la Recherche Scientifique, France

Reviewed by:
Nicole Renee Tartaglia, University of Colorado, USA
George T. Capone, Kennedy Krieger Institute, USA

***Correspondence:**
Nancy Raitano Lee, Department of Psychology, Drexel University, 3141 Chestnut Street, Stratton 119, Philadelphia, PA 19104, USA
nrl39@drexel.edu

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Executive functions (EF) are thought to be impaired in Down syndrome (DS) and sex chromosome trisomy (Klinefelter and Trisomy X syndromes; +1X). However, the syndromic specificity and developmental trajectories associated with EF difficulties in these groups are poorly understood. The current investigation (a) compared everyday EF difficulties in youth with DS, +1X, and typical development (TD); and (b) examined relations between age and EF difficulties in these two groups and a TD control group cross-sectionally. Study 1 investigated the syndromic specificity of EF profiles on the Behavior Rating Inventory of Executive Function (BRIEF) in DS ($n = 30$), +1X ($n = 30$), and a TD group ($n = 30$), ages 5–18 years. Study 2 examined age effects on EF in the same cross-sectional sample of participants included in Study 1. Study 3 sought to replicate Study 2's findings for DS by examining age-EF relations in a large independent sample of youth with DS ($n = 85$) and TD ($n = 43$), ages 4–24 years. Study 1 found evidence for both unique and shared EF impairments for the DS and +1X groups. Most notably, youth with +1X had relatively uniform EF impairments on the BRIEF scales, while the DS group showed an uneven BRIEF profile with relative strengths and weaknesses. Studies 2 and 3 provided support for fairly similar age-EF relations in the DS and TD groups. In contrast, for the +1X group, findings were mixed; 6 BRIEF scales showed similar age-EF relations to the TD group and 2 showed greater EF difficulties at older ages for +1X. These findings will be discussed within the context of efforts to identify syndrome specific cognitive-behavioral profiles for youth with different genetic syndromes in order to inform basic science investigations into the etiology of EF difficulties in these groups and to develop treatment approaches that are tailored to the needs of these groups.

Keywords: executive function, age, development, Trisomy 21, klinefelter syndrome, trisomy X syndrome, behavior, aneuploidy

Introduction

Over the past several decades, a great deal of progress has been made in characterizing the behavioral phenotypes associated with different genetic disorders—from disorders characterized by small microdeletions to full chromosomal aneuploidies (see Waite et al., 2014 for a review). While increases in knowledge about different behavioral phenotypes have been substantial, additional research is needed to isolate syndrome-specific characteristics from characteristics that are shared across syndromes. In the current investigation, we compare executive function (EF) profiles in Down syndrome (DS), sex chromosome trisomy (Klinefelter and Trisomy X syndromes; +1X), and typical development (TD). By comparing EF profiles in youth with these chromosomal trisomies not only to that of TD youth but also to one another, we aim to identify *etiologically-specific* characteristics of DS and +1X that may serve as more specific intervention targets for psychosocial and biomedical interventions.

The current investigation utilizes a caregiver report measure of EF, the Behavior Rating Inventory of Executive Function (BRIEF), to quantify the nature and severity of everyday EF difficulties in youth with DS and +1X. The BRIEF is a widely used measure in studies of DS and other developmental disorders. It is a useful tool for characterizing EF difficulties, particularly in populations such as DS and +1X, where there is considerable variability in cognitive ability levels. This cognitive variability can sometimes preclude the use of traditional laboratory measures for *all* participants in a given study. Thus, caregiver report can serve as an important index of real-world function and quality of life for participants with DS and +1X with a large range of ability levels. This has particular relevance for clinical trials assessment, as it permits a standard metric that can be used to evaluate change in functioning for all participants regardless of cognitive ability. Here we present cross-sectional data on the BRIEF that reveals a specific profile of ability and developmental trajectory in DS and +1X. Given that this measure is currently in use in a number of DS clinical studies underway, these data could serve as a benchmark for future work with this group.

In the sections that follow, we will describe the cognitive construct of EF and summarize one theoretical model that conceptualizes relations between different EF abilities. Then we will summarize the literature on the neuropsychology of DS and +1X, with a particular focus on what is known about EF abilities in these two groups. We will conclude with a description of the current study's research questions and hypotheses.

EF is an umbrella term used to describe a collection of higher-level cognitive abilities thought to be important for completing goals. A number of different abilities have been ascribed to this umbrella term, including working memory, planning, inhibition, and cognitive flexibility (Miyake et al., 2000; Lezak et al., 2004). EF abilities are thought to be important for various real world outcomes, including academic achievement (Blair and Razza, 2007) and work behavior (Ready et al., 2001).

Different conceptualizations of EF abilities exist. One conceptualization emphasizes the distinction between more cognitively-dominated executive processes, called cool EF (thought to be related to the functioning of the dorsolateral prefrontal cortex) and more affectively-heavy executive processes, called hot EF [thought to be related to the functioning of ventromedial prefrontal cortex (Metcalfe and Mischel, 1999; Zelazo and Muller, 2002)]. Examples of cool EF laboratory tasks include working memory tasks, such as backward digit span or spatial working memory, and planning tasks, such as the Tower of London. Examples of hot EF laboratory tasks include delay of gratification tasks and the Iowa Gambling task, among others. These hot EF tasks are thought to invoke the so-called "reward system"—that is, they require individuals to make choices that impact the size and/or immediacy of receiving a reward. Thus, they implicate motivation and affective systems more than traditional cool EF tasks. It has been proposed that different developmental disorders may be characterized by different cool and hot EF profiles (Zelazo and Muller, 2002). Thus, in the current study we seek to examine similarities and differences in the profile of cool and hot executive abilities in youth with DS and those with an additional X chromosome. In the next sections, we will summarize what is known about EF abilities and neuropsychological functioning more generally in individuals with DS and those with +1X.

Individuals with DS most often have IQs in the range of intellectual disability (standard scores <70); however, the neuropsychological phenotype in DS is more specifically characterized by language deficits in articulation and syntax (see Fowler et al., 1994 for a review) along with profound weaknesses in verbal short-term/working memory (see Baddeley and Jarrold, 2007 for a review). Additionally, DS is characterized by weaknesses in associative memory as well as motor delays (Pennington et al., 2003; Vicari, 2006). In contrast, some aspects of visual-spatial abilities, particularly visual-spatial short-term memory, and implicit learning have been reported to be mental age appropriate (Silverstein et al., 1992; Wang and Bellugi, 1994; Vicari et al., 2007). Furthermore, research examining behavioral difficulties in DS documents lower rates of problems compared to peers with other forms of intellectual or developmental disability (Dykens, 2007; though rates are higher than TD peers of similar chronological age).

Most studies of EF abilities in DS have examined one EF domain (e.g., working memory, inhibition) or have focused exclusively on more traditional cool, cognitively-mediated EF abilities within the laboratory setting. With a few exceptions (e.g., Pennington et al., 2003), these studies have documented deficits in the EF domains of inhibition, planning and problem-solving, cognitive flexibility/set-shifting, and working memory relative to typically-developing children matched on mental age or children with other forms of intellectual disability (Lanfranchi et al., 2004; Rowe et al., 2006; Lanfranchi et al., 2010; for a review, see Lee et al., 2011a, **Table 2**).

Abbreviations: EF, Executive Function; DS, Down syndrome; +1X, Participants with an additional X-chromosome (Klinefelter syndrome in males and Trisomy X syndrome in females).

In our prior work (Lee et al., 2011a) using the BRIEF—Preschool (BRIEF-P; Gioia et al., 2003), we found evidence for a specific DS profile relative to norms appropriate for mental-age. Specifically, this young sample of children with DS (mean age ∼6 years) demonstrated greater deficits in the so-called "cool" executive functions, such as working memory and planning, than the so-called "hot" executive functions, such as behavioral inhibition and emotional control (which were found to be commensurate with mental-age expectations but below chronological-age expectations). A more recent investigation by our group (Daunhauer et al., 2014) in which youth with DS were compared to MA-matched typically developing controls revealed a similar profile—that is, greater "cool" than "hot" EF difficulties. However, this study also documented inhibition difficulties (according to parent, but not teacher report) that exceeded mental age expectations, suggesting that the domain of behavioral inhibition may need to be investigated further in this group.

To the best of our knowledge, no studies have examined relations between age and EF abilities in youth with DS. This is particularly important for DS, as it is a disorder that is characterized by a slowing of cognitive development beginning in infancy (Hodapp and Zigler, 1990; Carr, 2005) as well as precocious onset of Alzheimer's disease in the fifth to sixth decades of life (Lott, 1982). Further, EF abilities show early emerging decline in adulthood in DS (Ball et al., 2006, 2008). Consequently, understanding the developmental stability of EF will be important for understanding the unfolding of the DS cognitive phenotype from childhood to young adulthood as well as informing studies seeking to identify individuals with DS who are at greatest risk for developing Alzheimer's disease later in life. We now turn to the literature on the neuropsychology of +1X.

Unlike DS, neither Klinefelter nor Trisomy X syndrome are typically associated with intellectual disability. Rather research suggests that the presence of an additional X chromosome is associated with approximately one standard deviation reduction in intellectual abilities relative to siblings or a well-matched typically developing control group (Polani, 1977). However, similar to DS, high rates of language-based learning disorders occur, including articulation difficulties, deficits in syntax, verbal memory weaknesses, and reading difficulties (for reviews, see Leggett et al., 2010; Lee et al., 2011b). Reports of behavioral and psychiatric difficulties in females and males with supernumerary X chromosomes have identified heightened rates of depressive and anxiety disorders in females (see Tartaglia et al., 2010 for a review) and heightened rates of attention and social difficulties in males (Tartaglia et al., 2006; Bruining et al., 2010).

EF difficulties, particularly on tasks with pronounced verbal demands, have been well-documented in Klinefelter syndrome. Deficits have been reported on tasks of verbal inhibition and verbal working memory as well as verbal fluency, the Trail Making Test, and both spatial working memory and planning tasks, such as the Stockings of Cambridge (Bender et al., 1993; Ross et al., 2009; Van Rijn et al., 2009; Lee et al., 2011c). For females with Trisomy X, limited data exist on EF abilities. However, the few studies that have examined EF abilities have documented weaknesses on tasks including the Wisconsin Card Sorting task and verbal fluency. Additionally, there have been reports of reduced attentional abilities relative to either siblings or typically-developing control participants (Bender et al., 1993, 2001). To our knowledge, no published papers have examined everyday EF abilities in males and females with an additional X chromosome. Moreover, no studies have examined the relations between age and EF difficulties in youth with sex chromosome trisomies. Thus, the current study will be the first report of its kind. In the section that follows, we summarize the questions asked by this investigation and the study hypotheses.

In the current investigation, we sought to answer two questions: (1) Are there unique EF profiles on the BRIEF for school-age children and adolescents (ages 5–18) with DS and those with +1X? (2) Do the relations between age and EF abilities in DS and +1X deviate from what is seen in TD (in this cross-sectional sample)?

Regarding question 1, we tested two competing hypotheses. Hypothesis 1 posited that there would be specificity for the profile of EF difficulties associated with DS and +1X (i.e., these disorders would be characterized by different patterns of scores on the BRIEF). Hypothesis 2 posited non-specificity—that is, the disorders would have a similar profile of scores, such that the two disorders cannot be discriminated based on BRIEF scores alone.

Regarding question 2, we tested three competing hypotheses. The first predicted developmental stability in EF problem behaviors for the DS and +1X groups—that is, the extent to which individuals with DS or +1X have EF difficulties in everyday life will be similar in magnitude in early childhood and young adulthood. This finding would mirror the pattern found in TD and would suggest that EF difficulties are present from early in development (prior to the ages studied here) and that the magnitude of these difficulties persists across the developmental period studied. The second hypothesis predicted developmental variability—that is, deviations in EF skills in youth with DS or +1X will differ at different stages of development. This may be reflected in a lessening of difficulties from early childhood to young adulthood such that deviations from TD decrease. Such a finding would be consistent with studies of other developmental disorders, such as specific language impairment, in which some research suggests that behavioral difficulties lessen as children age (St Clair et al., 2011). Conversely, increasing EF difficulties relative to typical peers may become apparent with age. This latter scenario is similar to that reported for youth with autism on the BRIEF, in which difficulties on several scales were found to show increasing impairment with age (Rosenthal et al., 2013).

These questions were investigated in three studies. Study 1 investigated the syndromic specificity of EF profiles for DS and sex chromosome trisomy using a traditional case-control design. Study 2 investigated age-effects on EF profiles for these two groups and contrasted findings with TD youth. Study 3 sought to replicate the DS age-effect findings from study 2 by examining age-EF relations in a large independent sample of youth with DS and TD representing a larger age range than included in Study 2.

Study 1: Contrasting the DS and +1X Profile on the BRIEF

Methods

Participants

Participants included 30 youth with DS recruited from two sites [University of Arizona ($n = 26$) and the National Institute of Mental Health (NIMH; $n = 4$)] and 30 youth with sex chromosome trisomy from the NIMH. Additionally, 30 TD youth from NIMH served as control participants. All participants were matched on chronological age and maternal education levels. We chose to match participants on chronological age (and not mental age as is often done in studies of youth with intellectual disability) because one of the primary goals of the larger investigation was to examine age effects on EF difficulties. Thus, we needed to match groups on age so that we could examine how the DS or +1X groups deviated from typically developing peers of the same age. To control for IQ differences among the groups, follow-up analyses were completed with nonverbal IQ covaried, as described further below. We also matched groups on maternal education levels in order to compare youth from similar family backgrounds (i.e., families with similar levels of educational achievement).

Participants with DS were recruited through family support groups local to the two sites and nationally. Participants were included in the current study if they had a confirmed medical diagnosis of DS according to parent report and had a complete BRIEF rating form (school age version).

Participants with sex chromosome trisomy (XXY and XXX) were recruited nationally with the help of parent advocacy groups to participate in a larger study of cognitive and brain development in youth with sex chromosome aneuploidies being conducted at the NIMH. The current sample represented a subsample of the larger group included in the NIMH study. To be included in the current sample, participants need to have a complete BRIEF form and also have a prenatal diagnosis of either Trisomy X syndrome or Klinefelter syndrome. This additional inclusion criterion was imposed on the sex chromosome trisomy group and not the DS group because unlike DS, many individuals with sex chromosome trisomies are unaware of their diagnosis (Boyd et al., 2010). Because there are not consistent physical dysmorphologies associated with the addition of an X-chromosome, many individuals go undiagnosed. As a result, samples that include postnatally-identified participants may be prone to include children with higher rates of learning and behavioral difficulties. This is believed to be the case, because often it is the presence of learning or behavioral difficulties that leads professionals to complete genetic testing in the absence of frank physical dysmorphologies. Thus, by excluding postnatally-diagnosed participants with +1X, we sought to provide a description of EF difficulties in this group that are not overly biased by participants who are having behavioral difficulties (that consequently led to the genetic testing and diagnosis). As a result, our descriptions of EF difficulties in this group may be more conservative than if we had included those with postnatal diagnoses. However, we deemed this as preferable to overstating the EF difficulties associated with sex chromosome trisomy.

TD participants were recruited through advertisements in the community and nationally. Prior to enrollment in the study, parents were interviewed about their child's development. Only participants without a history of developmental, learning, or psychiatric difficulties were included in the TD group.

For participants over the age of majority and with cognitive capacity to consent independently, written consent was obtained from the participant. For minors and those without capacity to consent independently, written consent was obtained from parents or legal guardians and the participant provided assent. The three studies included in this paper were reviewed and approved by the Institutional Review Board of each participating institution.

Demographic information about the three groups including age, sex, race, nonverbal IQ, and maternal education is summarized in **Table 1**. As shown in the table, groups did not differ on any of the demographic variables except for nonverbal IQ, which was expected. As will be seen in the Results section, IQ differences among the groups were controlled statistically in follow-up analyses and their effects on the study's findings are discussed.

Measures

Everyday executive function skill assessment

Parents of participants completed the school-age BRIEF form, developed for youth ages 5–18 years (Gioia et al., 2000). The school-age BRIEF has been utilized effectively in studies of DS,

TABLE 1 | Demographic information about the Down syndrome (DS), Sex Chromosome Trisomy (XXY & XXX; +1X), and Typically Developing (TD) control groups.

	DS ($n = 30$)			+1X ($n = 30$)			TD ($n = 30$)			
	M	*SD*	Range	*M*	*SD*	Range	*M*	*SD*	Range	F or X^2
Chron. Age	11.34	3.02	7–17	11.61	3.29	5–18	11.28	2.69	6–17	$F_{(2, 87)} < 1, p > 0.9$
Nonverbal IQ^	52.41	13.2	40–87	100.75	15.70	74–135	110.37	11.97	86–139	$F_{(2, 84)} = 150.56, p < 0.001$
Maternal Ed.	15.68	2.17	11–21	15.57	1.79	12–19	16.13	2.26	12–21	$F_{(2, 87)} < 1, p > 0.5$
	n	%		*n*	%		*n*	%		
Sex—male	15	50		15	50		15	50		$X^2 < 1, p > 0.9$
Race/Ethnicity—WNH	20	67		26	87		25	83		$X^{2(2)} = 4.1, p > 0.12$

^DS group n = 29 with Nonverbal IQ data; +1X group, n = 28 with Nonverbal data; missing data on 3 participants total for Nonverbal IQ. Chron. Age, Chronological Age; Maternal Ed, Year of Maternal Education; WNH, White, Non-hispanic.

and in the validation of the Arizona Cognitive Test Battery for DS, the measure correlated with laboratory tasks of EF and memory (e.g., CANTAB; Edgin et al., 2010). It has also been used effectively in studies of youth with sex chromosome aneuploidies (Janusz et al., 2011; Samango-Sprouse et al., 2015).

The BRIEF is an 86-item questionnaire that assesses EF behaviors in various domains. Caregivers describe their child's behavior using a 3-point Likert scale indicating how frequently their child engages in a given behavior (never = 1, sometimes = 2, often = 3). Higher scores denote greater problems. The BRIEF includes eight clinical scales: Inhibit, Shift, Emotional Control, Initiate, Working Memory, Plan/Organize, Organization of Materials, and Monitor. These scales were both theoretically and empirically derived. They are combined to create two indices: the Behavioral Regulation Index (Inhibition + Shift + Emotional Control Scales) and the Metacognition Index (Initiate + Working Memory + Plan/Organize + Organization of Materials + Monitor Scales). For the current investigation, T-scores were utilized to compare scores on the different scales across the groups. These T-scores were derived from the BRIEF manual. They are age and sex-adjusted and have a mean of 50; higher T-scores denote greater difficulties. Descriptions of the eight clinical scales are provided in **Table 2**.

While the BRIEF was not created to test differences in cool vs. hot EF difficulties, we believe that this is a useful classification system for two reasons. First, the BRIEF's two indices map roughly onto these constructs (e.g., the Metacognition Index measures common cool EF skills, including working memory and planning, while the Behavior Regulation index measures common hot EF skills, such as emotional control and inhibition). Second, this classification system has been a useful way to conceptualize the nature of EF difficulties in DS in our past work, albeit with the preschool BRIEF (Lee et al., 2011a; Daunhauer et al., 2014).

Nonverbal intelligence testing

Participants included in this study completed the Kaufman Brief Intelligence Test—Second Edition ($n = 26$; Kaufman and Kaufman, 2004), the Differential Ability Scales—Second Edition ($n = 4$; Elliott, 2007), or the Wechsler Abbreviated Scale of Intelligence Test ($n = 30$; Wechsler, 1999) per individual study protocols. We report on Nonverbal IQ rather than Full Scale (or Verbal) IQ in this study, because not all participants in Study 3 (which includes an independent sample of participants) completed an IQ test with a verbal portion. Thus, for consistency in reporting across the studies, we report only nonverbal IQ scores here. However, when using an estimate of overall intellectual ability as a covariate in analyses, we report the findings for nonverbal IQ but also note if they hold when Full Scale IQ is used as a covariate instead.

Statistical Analyses

Prior to completing primary analyses, the effects of sex on the eight BRIEF scales were evaluated to determine if it needed to be included in our models. This was done by running a series of independent samples *t*-tests within the three groups and comparing scores for males and females. No statistically significant sex differences were detected once the false discovery rate (FDR; Hochberg and Benjamini, 1990) correction was applied. The only sex differences that approached significance were found within the DS group for the Shift scale (where males had greater difficulties; $p = 0.02$) and the TD group on the Organization of Materials scale (where females had greater difficulties; $p = 0.04$).

Because these differences did not exceed thresholds for statistical significance, sex was excluded from the models and primary analyses were completed as follows. To examine differences in BRIEF profiles for the DS and +1X groups (as compared to TD controls), a series of mixed-model ANOVAs was completed. First, a 3 × 2 mixed-model ANOVA was completed with one between-subject factor (Group: DS vs. +1X vs. TD) and one within-subject factor (BRIEF Index: Behavior Regulation vs. Metacognition). This was followed by an additional mixed measure ANOVA in which the eight scales that constitute the Behavior Regulation and Metacognition indices were compared across groups. These ANOVAs were followed by tests of simple

TABLE 2 | Descriptions of BRIEF Clinical Scales belonging to the Behavioral Regulation and Metacognition Indices.

Scale name	Index	Description	Item examples
Inhibit	BR	Evaluates behaviors related to the ability to inhibit an impulse and stop behaviors when appropriate	Being fidgety or impulsive; getting more out of control than same-age peers
Shift	BR	Includes behaviors related to the ability to move from situation to situation or shift set	Resisting change in routines; becoming upset in new situations
Emotional control	BR	Evaluates behaviors related to the modulation of emotions	Having angry outbursts and getting upset easily
Working memory	MC	Assesses behaviors related to holding information online in memory in order to complete tasks with greater than one step	Having difficulty remembering multiple things to do or completing tasks with more than one step; having a short attention span
Plan/Organize	MC	Evaluates behaviors related to anticipating future events and organizing information and behavior to complete a goal	Having difficulty finding belongings, getting through routines, or initiating tasks
Initiate	MC	Examines generative behavior—i.e., beginning tasks and thinking of ideas/responses	Having difficulty getting tasks started; taking initiative
Organization of materials	MC	Measures how an individual organizes personal spaces and belongings	Leaving areas messy; having difficulties finding belongings

BR, Behavior Regulation; MC, Metacognition.

effects (using *t*-tests) when necessary and FDR correction was applied to adjust for multiple comparisons. Lastly, to account for differences in nonverbal IQ among the groups, ANCOVAs were run with nonverbal IQ covaried.

Results

Do Youth with DS and +1X Have Distinct Profiles on the BRIEF?

The 3 × 2 mixed-model ANOVA with one between-subject factor (Group: DS vs. +1X vs. TD) and one within-subject factor (BRIEF Index: Behavior Regulation vs. Metacognition) revealed a main effect of index [$F_{(1, 87)} = 9.04$, $p < 0.004$], a main effect of group [$F_{(2, 87)} = 22.37$, $p < 0.001$], but no group × index interaction [$F_{(2, 87)} = 2.04$, $p > 0.13$].

The main effect of index was such that scores tended to be lower (denoting fewer difficulties) on the Behavior Regulation Index than Metacognition Index overall. (However, it is important to note that this main effect appeared to be driven by the DS and TD groups, and not the +1X group. Specifically, when paired samples *t*-tests were run comparing the two indices for the three groups separately, the results were significant for the DS and TD groups (ps < 0.01) such that fewer problems with Behavioral Regulation were noted. In contrast, for the +1X group, these scores did not differ ($p = 0.9$), suggesting similar levels of impairment.)

The main effect of group was such that the TD controls had lower scores (fewer difficulties) overall than both of the aneuploidy groups (qs < 0.05; FDR corrected for 3 comparisons) which did not differ significantly from one another ($p > 0.70$). These results and those that follow are summarized in **Figure 1**.

To account for IQ differences among the groups, analyses were re-run with nonverbal IQ covaried. When nonverbal IQ was included as a covariate in a 3 × 2 mixed-model ANCOVA, the main effect of index was no longer significant, but the main effect of group remained [$F_{(2, 83)} = 13.01$, $p < 0.001$]. Tests of simple effects revealed that the TD group continued to outperform the +1X group ($q < 0.05$; FDR corrected for 3 comparisons). However, the DS group's index scores no longer differed from the TD or +1X groups. These analyses were also run with Full Scale IQ covaried and results were largely the same.

Next the eight BRIEF scales were submitted to a 3 × 8 mixed-model ANOVA with one between-subject factor (Group: DS vs. +1X vs. TD) and one within-subject factor (Scale: the eight BRIEF scales). Results revealed a main effect of scale [$F_{(5.06, 440.10)} = 5.39$, $p < 0.001$], a main effect of group [$F_{(2, 87)} = 21.57$, $p < 0.001$] and a group X scale interaction [$F_{(10.12, 440.10)} = 6.87$, $p < 0.001$] (Note: Because the assumption of sphericity was violated, the Greenhouse Geisser adjustment was applied and the degrees of freedom were adjusted).

Tests of simple effects (FDR adjusted for 24 comparisons) revealed that TD controls had lower scores (fewer difficulties) than the +1X group on all of scales (qs < 0.05; FDR corrected). The TD group also differed from the DS group on all scales (qs < 0.05; FDR corrected) except for the Organization of Materials scale ($p = 0.87$). In contrast, for the DS and +1X groups, only two scales differed significantly when FDR correction was applied: Emotional Control (DS < +1X; fewer problems) and Monitor (DS > +1X; more problems). When the unadjusted *p*-values were considered, the DS group had lower scores (denoting

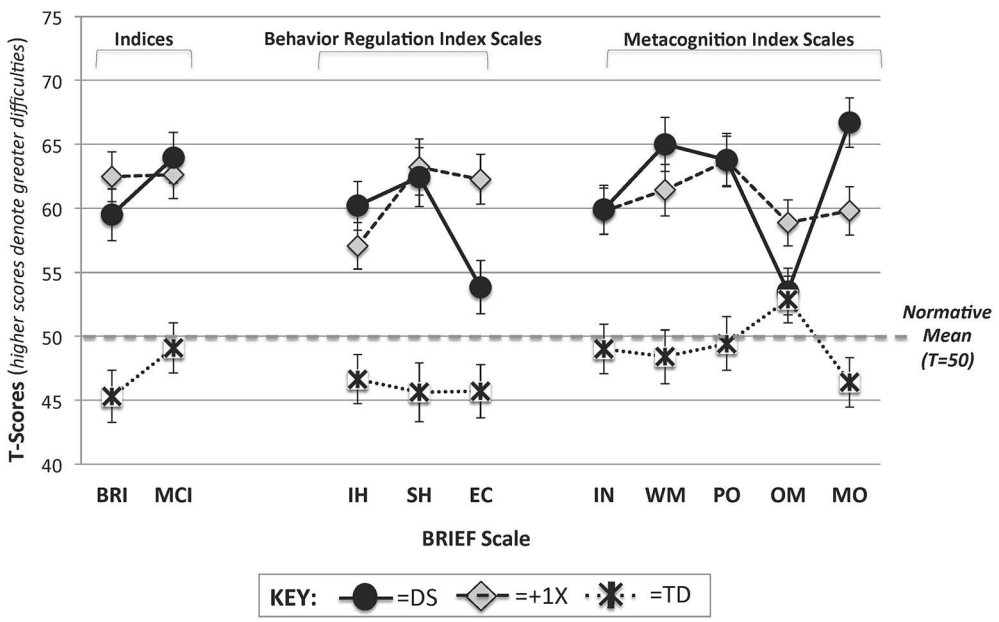

FIGURE 1 | BRIEF profiles for the DS, +1X, and TD groups. T-Scores on the BRIEF Indices and Scales are provided for the DS (solid black circles with solid line), +1X (gray diamonds with long dotted line), and TD (asterisks with small dotted line) groups. Note that the normative mean is 50 and that greater T-scores denote greater levels of difficulty. BRI, Behavior Regulation Index; MCI, Metacognition Index; IH, Inhibit; SH, Shift; EC, Emotional Control; IN, Initiate; WM, Working Memory; PO, Plan/Organize; OM, Organization of Materials; MO, Monitor.

fewer difficulties) than the +1X group on the Organization of Materials Scale ($p = 0.04$) as well.

In order to examine the pattern or profile of scores *within* each group, differences in performance on the eight scales were evaluated (and FDR correction was applied for 24 comparisons; 8 for each group). This was done by calculating the group mean on the eight scales and then comparing each scale to this value using paired samples *t*-tests. For the TD group, only the Organization of Materials scale was significantly higher than the overall mean ($q < 0.05$), indicating that this was an area of relative weakness. For the +1X group, there were no significant differences between the individual scales and the overall mean. In contrast, the DS group demonstrated several peaks and valleys in their profile. The following scores were higher than the mean (denoting relative weaknesses): Working Memory and Monitor. In contrast, the following scores were lower than the mean (denoting relative strengths): Emotional Control and Organization of Materials.

In order to control for nonverbal IQ differences among the groups, a 3 × 8 mixed-model ANCOVA was run with nonverbal IQ included as a covariate. With nonverbal IQ in the model, the main effect of group [$F_{(2,\ 83)} = 11.96$, $p < 0.001$] and the Group X Scale interaction [$F_{(10.38,\ 430.56)} = 2.36$, $p < 0.01$] remained significant (Note: Because the assumption of sphericity was violated, the Greenhouse Geisser adjustment was applied and the degrees of freedom were adjusted). Tests of simple effects (FDR adjusted for 24 comparisons) revealed that the TD group received lower scores (denoting fewer difficulties) than the +1X group on all scales (qs < 0.05) except for Organization of Materials. For the DS group, no scales differed significantly from the +1X or TD groups with nonverbal IQ included as a covariate in the model. However, when unadjusted *p*-values were considered, the DS group's score on the Emotional Control scale continued to be lower (denoting fewer difficulties) than +1X group ($p = 0.02$) while their scores on the Working Memory ($p = 0.03$), Monitor ($p = 0.03$), and Inhibit ($p = 0.04$) scales were higher than the TD group (denoting greater difficulties). Lastly, when Full Scale IQ was covaried instead of nonverbal IQ, the main effect of group and group x scale interaction remained statistically significant. However, the tests of simple effects revealed slightly different results. While Emotional Control continued to be significantly lower (denoting fewer difficulties) in the DS than the +1X group (uncorrected $p < 0.05$), the differences noted for the DS and TD groups described above were not statistically significant.

Summary and Discussion: Study 1

In this study, we sought to evaluate the specificity of the DS and +1X profiles on the BRIEF by contrasting scores with one another and a TD control group matched on chronological age and maternal education levels. First, to evaluate the profile of differences associated with hot vs. cool EF abilities, we contrasted scores on the Behavior Regulation (which evaluates behaviors that are typically associated with hot EF abilities) and Metacognition (which evaluates behaviors that are typically associated with cool EF abilities) indices of the BRIEF. Replicating our prior findings using the BRIEF-P (Lee et al., 2011a; Daunhauer et al., 2014), we find that participants with DS received higher scores (denoting greater difficulty) on the Metacognition Index than the Behavior Regulation Index of the BRIEF, consistent with greater cool EF difficulties. However, this pattern of scores was not specific to DS, but rather was similar to what was found in the TD group. While there was no group x condition interaction for this analysis, it is important to note that the pattern of index scores for DS was different than the pattern found for the +1X group. Specifically, there was no significant difference between the two indices for this group ($p = 0.9$), suggesting similar levels of difficulties in these two EF domains for youth with +1X.

When the eight scales were compared for the groups of youth with DS and +1X, evidence for both shared and unique features were found. Regarding the shared features, the DS and +1X groups demonstrated similar degrees of EF difficulty on the following scales (all of which were elevated relative to TD controls): Inhibit, Shift, Initiate, Working Memory, and Plan/Organize. Additionally, the groups did not differ on Organization of Materials scale; however, the +1X group's scores were elevated relative to TD controls, while the DS and TD control scores did not differ ($p = 0.87$).

With regard to differences/unique features, the DS group demonstrated greater levels of impairment than +1X group on the Monitor scale; the opposite was true for the Emotional Control scale where the +1X group demonstrated greater levels of impairment. In addition to these two differences, the greatest evidence for *specificity* of BRIEF profiles for the +1X and DS groups came from an examination of the pattern of scores across the BRIEF scales. While the +1X group had a relatively flat profile of scores on the BRIEF (denoting similar levels of difficulties on the different scales), the DS group demonstrated a much more variable profile. Specifically, weaknesses were noted on the Working Memory and Monitor scales while strengths were noted on the Emotional Control and Organization of Materials scales.

Study 2: Contrasting Age-effects on BRIEF Scales for the DS +1X Groups

In this cross-sectional study, we examined the relations between age and EF difficulties in youth with DS and those with +1X. In particular, we sought to test hypotheses about the stability or variability in the severity of EF difficulties for youth with DS and +1X relative to youth with TD.

Methods

Participants

Participants were the same as those included in Study 1. See Method section above and **Table 1** for details.

Measures

Everyday executive function skill assessment

Again, the BRIEF was used. However, unlike Study 1, raw scores on the BRIEF scales were used as dependent variables rather than age- and sex-adjusted T-scores. Raw scores were preferred over T-scores so that relations between age and total difficulties (unadjusted for age) could be evaluated. In order to allow easy comparison across scales, mean item severity scores were calculated for the eight scales. Specifically, scores on the

items included in each scale were totaled and were divided by the number of items in that scale.

Statistical Analyses

To examine age-related differences in scores among the DS, +1X and TD groups, hierarchical linear regression was used, with three steps: (1) age, (2) group, and (3) age X group interaction. This last step was used to evaluate whether relations between age and EF difficulties varied among the three groups. If this last step was significant, then Pearson correlation coefficients between age and raw scores for each of the pairs (TD vs. DS; TD vs. +1X; +1X vs. DS) were contrasted using a Fishers-R-to-Z transformation.

Results

Are There Similar Relations between Age and BRIEF Scale Ratings for Youth with DS, +1X, and Those with Typical Development?

Results of hierarchical linear regressions evaluating the effects of age, group, and their interaction for the eight clinical scales of the BRIEF are summarized in **Table 3** and in **Figure 2**. For six of the eight scales, the effects of age did not appear to vary as a function of group—i.e., the magnitude of the relationship between BRIEF scale raw mean scores and age was similar for the TD, DS, and +1X groups. However, for two of the scales—Initiate and Plan/Organize—there were age X group interactions in the prediction of scores. In both cases, the +1X group's difficulties on the BRIEF appeared to be more severe at older ages while the DS and TD groups' EF difficulties were less severe at older ages (Fisher's $Z \geq 1.96$, ps < 0.05).

Summary and Discussion: Study 2

In the current cross-sectional study, we evaluated the relations between age and EF performance on the BRIEF in youth with DS, +1X, and those with TD. Largely, there was support for similar relations between age and scale scores for the DS and TD groups, lending support for the developmental stability hypothesis for the DS group. As can be seen in **Figure 2**, the trend in the data for the DS group was for fewer difficulties with increasing age. This paralleled the findings in the TD group. Given the small sample size and the fact that the developmental stability hypothesis is essentially supporting the null hypothesis, Study 3 was completed with a larger independent sample to determine if these results could be replicated across a slightly larger age range (ages 4–24 years) and with a larger group.

TABLE 3 | Hierarchical Linear Regression Results Using Age, Group, and the Age*Group Interaction to Predict BRIEF Scale Raw Scores (Means).

DV	Step	IVs	*R*	R^2 Change	*F* change	Df	*p*
Inhibit	1	Age	0.32	0.11	10.34	1, 88	0.00
	2	Group	0.48	0.12	13.96	1, 87	0.00
	3	Age*Group	0.48	0.00	0.04	1, 86	0.84
Shift	1	Age	0.00	0.00	0.00	1, 88	0.98
	2	Group	0.50	0.25	28.90	1, 87	0.00
	3	Age*Group	0.52	0.02	2.50	1, 86	0.12
Emotional control	1	Age	0.13	0.02	1.6	1, 88	0.21
	2	Group	0.53	0.26	30.9	1, 87	0.00
	3	Age*Group	0.53	0.00	0.20	1, 86	0.66
Initiate	1	Age	0.04	0.00	0.13	1, 88	0.72
	2	Group	0.38	0.14	14.10	1, 87	0.00
	3	Age*Group	0.46	0.07	7.64	1, 86	0.01
Working memory	1	Age	0.16	0.03	2.25	1, 88	0.14
	2	Group	0.43	0.16	17.06	1, 87	0.00
	3	Age*Group	0.45	0.02	1.64	1, 86	0.20
Plan/Organize	1	Age	0.01	0.00	.01	1, 88	0.93
	2	Group	0.46	0.22	23.82	1, 87	0.00
	3	Age*Group	0.51	0.05	5.25	1, 86	0.02
Organization of materials	1	Age	0.15	0.02	1.89	1, 88	0.17
	2	Group	0.28	0.06	5.26	1, 87	0.02
	3	Age*Group	0.29	0.01	0.81	1, 86	0.37
Monitor	1	Age	0.10	0.01	1.07	1, 88	0.30
	2	Group	0.44	0.18	19.45	1, 87	0.00
	3	Age*Group	0.46	0.02	1.94	1, 86	0.17

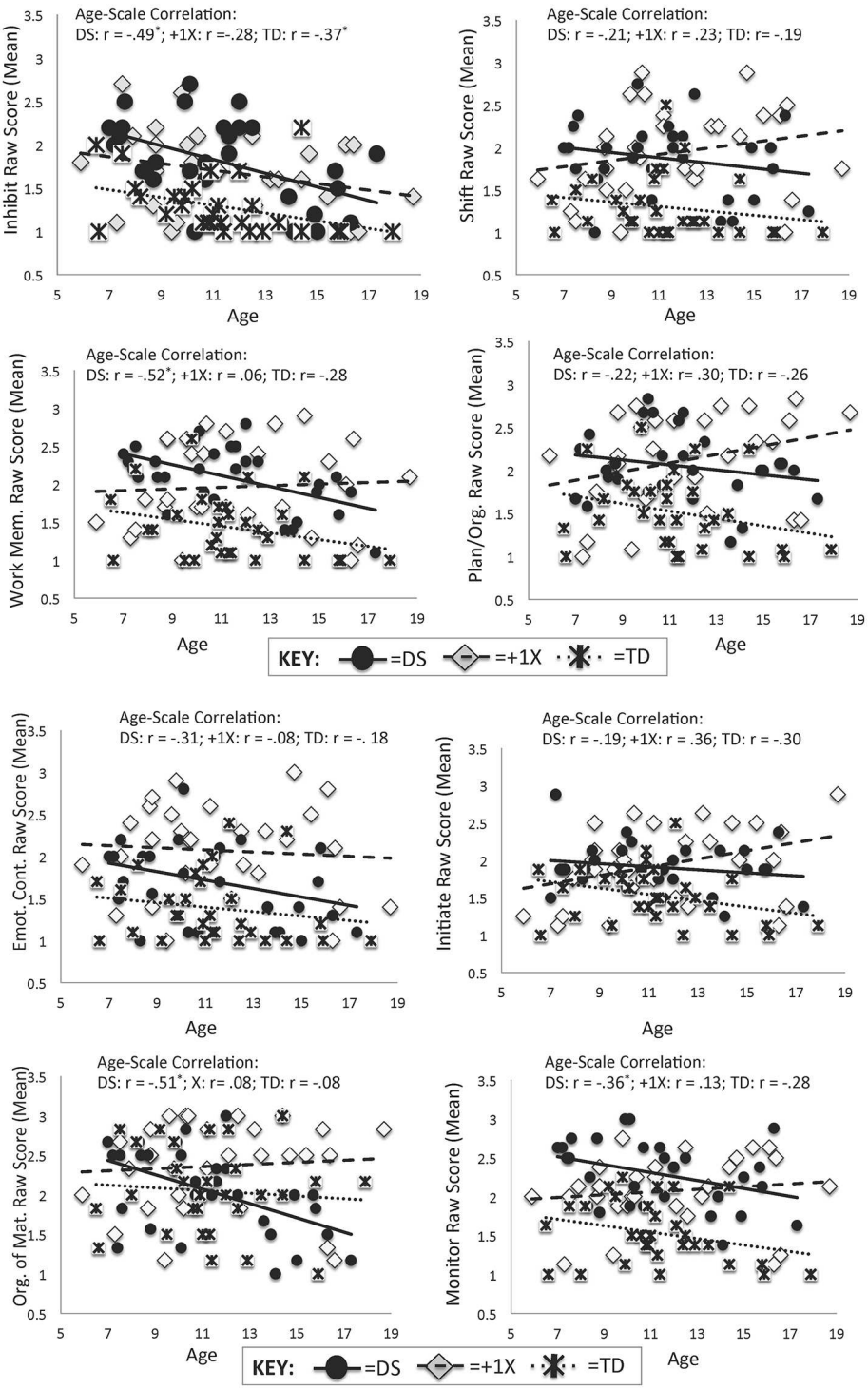

FIGURE 2 | Scatterplots for Age and BRIEF Scales by Group. Scatterplots display the relations between age and raw scores on the BRIEF scales for the DS (solid black circles with solid line), +1X (gray diamonds with long dotted line), and TD (asterisks with small dotted line) groups. Note that higher scores denote greater difficulties.

For the +1X group, the findings were mixed—for six of the scales, there was support for developmental stability. However, for the Initiate and Plan/Organize scales, there appeared to be support for developmental variability. In both cases, the trend in the data was for caregivers' ratings of EF difficulties to increase with increasing age (denoting greater difficulties later). However,

given the cross-sectional nature of these data as well as the small sample size, these findings must be interpreted cautiously. Further discussion and interpretation of these findings will be provided in the General Discussion section.

Study 3: A Replication Study of Age-BRIEF Relations in the DS and TD Groups

In this cross-sectional study, we sought to replicate our earlier findings of developmental stability on the BRIEF with a larger independent sample of youth with DS ($n = 85$) and a TD control group ($n = 43$).

Methods

Participants

Participants included 85 youth with DS recruited from three sites: the University of Arizona ($n = 36$), Colorado State University ($n = 31$), and NIMH ($n = 18$). A total of 43 typically developing control participants matched on chronological age and maternal education levels were recruited from two sites: the University of Arizona ($n = 13$) and the National Institute of Mental Health ($n = 30$). Rationale for matching on chronological age (rather than mental age) can be found in the Method section of Study 1.

Demographic information about the two groups including age, sex, race, nonverbal IQ, and maternal education is summarized in **Table 4**. As shown in the table, groups did not differ on any of the demographic variables except for nonverbal IQ, which was expected and similar to the findings from the previous two studies.

Measures

Everyday executive function skill assessment

Unlike the prior study in which the school-age BRIEF was utilized, the current study included participants with either the school-age BRIEF, developed for participants age 5–18 or the preschool BRIEF (BRIEF-P), developed for participants age 2–5. The inclusion of the two versions of the BRIEF permitted combining data collected on participants with DS over a large age range who participated in studies at the three sites listed above.

Despite differences in targeted age range, there are a number of shared items on the BRIEF and BRIEF-P that permitted the creation of composite scores that could be used regardless of the version of the BRIEF that was administered. As will be described in further detail below (under the subheading, "Creation of Study-derived BRIEF Composites"), 41 items were extracted from the two versions of the BRIEF to create five composite scores that mapped onto the five indices included on the preschool BRIEF—the Emotional Control, Inhibit, Shift, Working Memory, and Plan/Organize indices.

The version of the BRIEF administered was determined by site protocol. For participants with DS recruited at Colorado State University and the National Institute of Mental Health, mental age was used to determine the version of the BRIEF that was administered, consistent with prior publications from our labs (Lee et al., 2011a; Daunhauer et al., 2014). Thus, even if participants were >5 years of age, they were given the preschool BRIEF if their mental age was between the ages of 2 and 5 years. Similarly, if they were greater than 18 years of age but their mental age was between the ages of 5 and 18, they were given the school-age BRIEF. Participants with DS recruited from the University of Arizona and all but one control participant (who was over the age of 18) were given the chronological age appropriate version of the BRIEF. While the correct version of the BRIEF for the one typically developing participant over the age of 18 (age 22 years) was technically the BRIEF-A (adult), no one else in the study had data on the BRIEF-A. Thus, we asked that this adult request that his/her parents complete the school age BRIEF (given that we were only using raw scores on certain items for this particular study, as described below).

In total, 44 participants with DS and 30 TD controls received the BRIEF; 41 participants with DS and 13 TD controls received the BRIEF-P. As described earlier, the BRIEF has 86 items; the BRIEF-P has 63 items. Both versions use the same 3-point Likert scale with which caregivers indicate how frequently their child engages in a given behavior (never = 1, sometimes = 2, often = 3). Higher scores denote greater problems on both instruments.

Unlike the BRIEF which includes eight clinical scales (see **Table 2** for details), the BRIEF-P includes five clinical scales that

TABLE 4 | Demographic information about the Down syndrome (DS) and typically developing (TD) control groups.

	DS ($n = 85$)			TD ($n = 43$)			
	M	***SD***	**Range**	***M***	***SD***	**Range**	**X^2 or T-stat**
Chron. Age	12.3	5.03	4–24	12.35	5.41	4–22	$t_{(126)} < 1, p > 0.95$
Nonverbal IQ^	52.53	14.72	24–112	106.4	12.22	67–131	$t_{(124)} = 20.59, p < 0.001$
Maternal Ed.	15.69	2.34	6–21+	16.02	2.10	13–21+	$t_{(186)} < 1, p > 0.43$
	n	**%**		***n***	**%**		
Sex—male	48	57		20	47		$X^2 < 1.2, p > 0.28$
Race/Ethnicity—WNH*	52	63		28	65		$X^2 < 1, p > 0.85$

^*DS group n = 83, missing data on 2 participants.*

**DS group n = 82, missing complete race and ethnicity information on 3 participants.*

Chron. Age, Chronological Age; Maternal Ed = Year of Maternal Education; WNH, White Non-hispanic.

are a subset of the eight from the BRIEF. These include Inhibit, Shift, Emotional Control, Working Memory, and Plan/Organize. These scales were also theoretically and empirically derived. They are combined to form three indices: the Inhibitory Self-Control Index (Inhibition + Emotional Control Scales), Flexibility Index (Shift + Emotional Control Scales), and Emergent Metacognition Index (Working Memory + Plan/Organize Scales).

Creation of study-derived BRIEF composites

In order to examine age effects using the two instruments, shared items from the BRIEF and BRIEF-P were extracted for each participant and composites were created. Because there are fewer clinical scales on BRIEF-P and all five of its scales are also found on the BRIEF (which has three additional scales), item composites were created based upon the item's scale on the BRIEF-P (i.e., if an item was a part of the Working Memory scale on the BRIEF-P, it was included in the Working Memory composite in this scheme). Thus, the current study included five composites, which mapped onto the five clinical scales from the BRIEF-P: Inhibit, Shift, Emotional Control, Working Memory, and Plan/Organize. See **Table 2** for descriptions of the types of items that are included in these five scales.

Scores for the five composites were created by calculating the average item rating across all items included in that composite. Items were included in the composites created here if they were identical on the BRIEF and BRIEF-P or if the content of the words varied slightly but the targeted behavior was the same. For example, the BRIEF item "has to be closely supervised" was considered equivalent to the BRIEF-P item "has to be more closely supervised than similar playmates." Similarly, the BRIEF item "is fidgety," was considered equivalent to the BRIEF-P item "is fidgety, restless, or squirmy." In total, 41 items were extracted from the two instruments. Twenty had identical wording and 21 had similar wording.

The BRIEF and BRIEF-P items that were included in the five composites created for this investigation are summarized below. The BRIEF item is listed first, followed by the BRIEF-P item (which has a P with it).

The *Emotional Control Composite* included items: 1 & 1P, 7 & 6P, 25 & 16P, 26 & 21P, 45 & 36P, 62 & 31P, 64 & 26P, 70 & 11P.

The *Inhibit Composite* included items: 34 & 3P, 38 & 18P, 42 & 33P, 44 & 43P, 78 & 13P, 54 & 52P, 55 & 54P, 59 & 60P, 63 & 38P, 81 & 23P, 82 & 28P.

The *Shift Composite* included items: 6 & 5P, 12 & 15P, 23 & 45P, 80 & 35P.

The *Plan/Organize composite* included items: 10 & 9P, 28 & 39P, 33 & 14P, 67 & 44P, 69 & 34P, 75 & 19P, 86 & 24P.

The *Working Memory Composite* included items: 2 & 2P, 9 & 61P, 17 & 12P, 21 & 22P, 24 & 27P, 27 & 32P, 32 & 37P, 37 & 42, 47 & 51P, 57 & 59P, 83 & 47P.

To demonstrate the similarities in the composite created for this study and the raw score for the corresponding clinical scale on the BRIEF or BRIEF-P, Pearson correlation coefficients were run. For the BRIEF, the correlations between the study-generated composites and raw totals were as follows for the Inhibit, Shift, Emotional Control, Working Memory, and Plan/Organize scales, respectively: 0.94, 0.94, 0.99, 0.98, 0.75. For the BRIEF-P, the correlations were as follows, respectively: 0.99, 0.94, 0.99, 0.97, 0.96.

Nonverbal intelligence testing

Participants at the three sites were given different intelligence tests per individual study protocols. These included the Leiter International Performance Scale—Revised ($n = 31$; Roid and Miller, 1997), the Kaufman Brief Intelligence Test—Second Edition ($n = 55$; Kaufman and Kaufman, 2004), the Differential Ability Scales—Second Edition ($n = 12$; Elliott, 2007), the Wechsler Abbreviated Scale of Intelligence ($n = 29$; Wechsler, 1999), and the Wechsler Preschool and Primary Scale of Intelligence—Third Edition ($n = 1$; Wechsler, 2002).

Statistical Analyses

Similar to Study 2, age effects on the five BRIEF composites were examined using hierarchical linear regression with three steps: (1) age, (2) group, and (3) age X group interaction. This last step was the step used to evaluate whether relations between age and EF difficulties varied for the DS and TD groups.

Results

Results of regression analyses can be found in **Table 5**. As can be seen, there were no age X group interactions for any of the regression equations, consistent with findings of Study 2. Rather, relations between age and BRIEF scores were similar for the DS and TD groups.

Summary and Discussion: Study 3

Taken together, the results of Study 3 provide additional support for stability in the DS profile on the BRIEF from early childhood to young adulthood. Specifically, a similar relationship between age and the BRIEF EF composite scores was found for the DS and control groups. For all composites except the Inhibit composite, age effects were non-significant and there were no age X group interactions, indicating that neither group's BRIEF scores were strongly predicted by age. For the Inhibit composite, significant age effects were found, such that inhibit scores improved (decreased) with age. However, these findings were similar in the DS and control groups, as evidenced by the lack of a group X age interaction. Despite the lack of an interaction effect, it is worth noting that the DS group's higher scores on the Inhibit scale paired with parallel rates of decreasing difficulties as compared to controls suggests that these difficulties may continue to lessen into the mid 20s to early 30s and eventually reach the level of the TD group, albeit at a much older age. This hypothesis would need to be confirmed with an older and/or longitudinal sample.

General Discussion

In this paper, we asked two primary questions: (1) Are there unique EF profiles on the BRIEF for school-age children and adolescents (ages 5–18) with DS and those with +1X? (2) Do the relations between age and EF abilities in DS and +1X deviate from what is seen in TD?

TABLE 5 | Hierarchical linear regression results using Age, Group, and the Age*Group interaction to predict BRIEF scale raw scores (Means).

DV	Step	IVs	*R*	R^2 Change	F change	df	*p*
Inhibit Composite	1	Age	0.24	0.06	7.65	1, 126	0.01
	2	Group	0.58	0.28	51.93	1, 125	0.00
	3	Age*Group	0.58	0.00	0.09	1, 124	0.76
Shift Composite	1	Age	0.01	0.00	0.01	1, 126	0.93
	2	Group	0.44	0.19	29.29	1, 125	0.00
	3	Age*Group	0.44	0.00	0.56	1, 124	0.46
Emotional Control Composite	1	Age	0.14	0.02	2.36	1, 126	0.13
	2	Group	0.31	0.08	10.66	1, 125	0.00
	3	Age*Group	0.32	0.01	0.66	1, 124	0.42
Work. Mem. Composite	1	Age	0.09	0.01	1.06	1, 126	0.31
	2	Group	0.67	0.44	99.12	1, 125	0.00
	3	Age*Group	0.67	0.00	0.15	1, 124	0.70
Plan/Organize Composite	1	Age	0.01	0.00	0.01	1, 126	0.93
	2	Group	0.51	0.26	44.62	1, 125	0.00
	3	Age*Group	0.52	0.00	0.39	1, 124	0.54

With regard to the first question, that of syndromic specificity of BRIEF profiles, we find some evidence for specificity and some for overlap. Specifically, there were several scales on the BRIEF in which the DS and +1X groups were similarly impaired. These included the Inhibit, Shift, Initiate, Working Memory, Plan/Organize, and Organization of Materials scales. In contrast, the DS group received lower scores (denoting fewer difficulties) on the Emotional Control scale while the +1X group received lower scores on the Monitor scale.

Interestingly, the greatest difference between the two groups appears to be in the pattern or profile of scores rather than the absolute values of the scores. More specifically, the +1X group showed a relatively flat profile of scores on the BRIEF—that is, there was little variation in scores across the eight BRIEF scales. In contrast, the DS group had several peaks and valleys in their scores. In particular, scores on the Working Memory and Monitor scales were peaks, denoting greater difficulties, while the Organization of Materials and Emotional Control scales were valleys, denoting relative strengths. These two relative strengths are noteworthy.

First, the finding of relatively lower levels of difficulty with Emotional Control fits with studies suggesting that youth with DS have lower rates of psychiatric difficulties than youth with other developmental disabilities (Dykens, 2007). This also fits with our prior studies suggesting that youth with DS have fewer hot than cool EF difficulties (Lee et al., 2011a; Daunhauer et al., 2014). However, it is important to note that while this is a relative strength in DS, it is not an absolute strength. Rather, difficulties with emotional control are higher in DS than those found in same age typically developing peers (analogous to rates of psychiatric difficulties). Second, relatively lower difficulties on the Organization of Materials scale (which were essentially commensurate with the TD group) may relate to anecdotal reports suggesting that some people with DS are very concerned with the organization of their belongings and prefer to have things be "just so." We have observed this clinically and have had parents mention that their children can be insistent on the order/organization of particular things in their homes. Despite the speculative nature of these observations, it may be helpful to emphasize that this particular set of skills should be viewed as a relative strength and thus may prove useful in designing interventions aimed at improving organization and planning as it relates to cognitively demanding academic tasks. This is particularly relevant for DS, as two of their greatest weaknesses on the BRIEF were on the Working Memory and Monitor scales. Both of these scales assess abilities that are important for academic outcomes, and thus, developing strategies to improve these skills may be a target for future investigations.

For youth with +1X, executive difficulties appear to be quite significant and uniform. It is noteworthy that this group's mean nonverbal IQ score was over three standard deviations higher than the DS group's, but the group's scores on seven of the eight BRIEF scales were similarly or more impaired. Furthermore, when nonverbal IQ was controlled for in ANCOVA analyses, group differences between the +1X and TD groups remained on seven of the eight scales (with FDR correction for multiple comparisons). This was not the case for the DS group. Thus, it appears that many of the everyday EF difficulties that accompany +1X are well in excess of IQ reductions associated with syndrome.

Furthermore, unlike youth with DS, difficulties with emotional control appear to be related to Klinefelter and Trisomy X syndromes, suggesting that future examinations should probe hot executive difficulties in sex chromosome trisomies in greater detail. This finding fits with studies indicating higher rates of mood and attentional difficulties for females and males with sex chromosome trisomies, respectively (Tartaglia et al., 2006, 2010). Thus, the current results highlight the importance of close monitoring of mood and attentional difficulties for youth with Klinefelter and Trisomy X syndromes. This is especially

important given that this study included only prenatally diagnosed participants with sex chromosome aneuploidies (a strength of the current research). Had we permitted the inclusion of participants with postnatally diagnosed sex chromosome aneuploidies, we suspect that we would have found even greater levels of difficulties.

With regard to the second question about the developmental stability of EF difficulties in youth with DS and those with +1X, there was consistent support for developmental stability in the DS group and mixed findings in the +1X group. For the DS group, this question was addressed both in studies 2 and 3 with two independent samples. Results were similar across the two studies—namely, the degree of EF difficulties on all domains of the BRIEF examined were similar to that of TD controls across the study's age range (5–18 years in Study 2 and 4–24 years in Study 3).

In the group of youth with +1X, the overall trend in the data supported the developmental stability hypothesis. However, two scales—Initiate and Plan/Organize—were associated with greater deviations from typical peers (and DS peers) with increased age. These findings must be interpreted very cautiously for several reasons. First and most importantly, this is a cross-sectional study. Thus, we cannot suggest that these skills are worsening over time. There could be a bias in our sample such that more impaired youth tend to be older. However, this seems unlikely given that not all scales were associated with greater difficulties at older ages. To control for any possible IQ confounds in our sample (i.e., a possible confound in which older participants had lower IQ scores), partial correlation analyses were run between each of the BRIEF scales and age with the effects of nonverbal IQ removed. For the three groups, the direction of the relations between age and BRIEF raw scores remained the same (positive correlations between age and both Initiate and Plan/Organize scores in the +1X group and negative correlations for the DS and TD groups).

Second, increases in perceived problems may relate to increased expectations that parents place on older youth with +1X that are not placed on older youth with DS, for example, possibly due to the IQ differences between the groups. It may be that as youth with +1X age, expectations increase and parent ratings reflect this. Future research investigating EF difficulties with laboratory tests may help rule out or confirm this possibility.

Existing research on the relations between age and everyday EF difficulties in other developmental disabilities is limited. One set of investigators (Rosenthal et al., 2013) examined these relations in a cross-sectional sample of youth with autism spectrum disorders and reported a worsening of EF difficulties on the Working Memory, Initiate, and Organization of Materials scales with age (using BRIEF norms and a cross-sectional sample). These findings fit with those found for +1X and contrast with those found for the DS group.

Our findings of stability in the degree of EF difficulties over the course of childhood and into young adulthood in DS may be a specific feature of the behavioral phenotype over the age range studied. This will need to be examined in future research with longitudinal samples. Additionally, it will be important to examine the stability of EF scores on the BRIEF (and using laboratory instruments) across the lifespan in DS, as the heightened rates of precocious Alzheimer's in DS suggest that the fifth and sixth decades of life are times in which EF difficulties may change for some individuals with DS. Furthermore, more research is needed prior to school age to understand the development of EF difficulties from infancy through the preschool years. Thus, it will be crucial to include these age groups in future research.

We now turn to discussing the limitations of our studies. For Study 1, we were limited by small sample sizes. Thus, we may have been underpowered to detect more subtle differences between the DS and +1X groups on the BRIEF scales. Furthermore, given our small samples, we were not able to thoroughly investigate possible sex differences within the groups. While our preliminary investigation of this suggested no large sex differences in male and female scores between the groups, the small samples may have resulted in our being underpowered to detect these differences. We were most concerned about the impact of sex differences within the +1X group, given that males with Klinefelter syndrome and females with Trisomy X syndrome are often considered separately in the literature. However, the overall trend in the BRIEF data examined here was for very similar scores on the BRIEF scales for males and females with +1X. This is consistent with our findings from an earlier study of language difficulties in this population (Lee et al., 2012). Lastly, an additional limitation of Study 1 is that the IQ scores for the DS and +1X groups were markedly different (and different from that of the TD controls). While this likely contributed to differences in performance on the BRIEF, it is important to note that the biggest difference in EF for these two groups appeared when the profile of EF difficulties on the BRIEF was examined *within* each group (i.e., when each BRIEF scale score was compared to the mean of the scale scores for that group). Thus, this set of analyses was not concerned with absolute differences between the groups but rather the profile of scores within each group. Moreover, given the IQ differences between the DS and +1X groups, it is especially noteworthy that the +1X group had EF difficulties that were similar to (or even exceeded) the DS group. This finding should underscore the degree of EF difficulties encountered by youth with Klinefelter and Trisomy X syndromes and encourage future research on the nature of these EF difficulties.

For Studies 2 and 3, the greatest limitation was the cross-sectional research design. We recognize this weakness, but see these studies as first steps forward in describing trajectories of everyday EF difficulties in DS and +1X. While our +1X sample was relatively small, our sample of youth with DS is one of the largest studied with this well-known and validated measure. Thus, these results add to our understanding of the EF profile and possible developmental trends in both DS and +1X.

Clearly, longitudinal studies are needed and should follow this study to confirm (or refute) these findings. Moreover, as stated earlier, it will be important to examine the stability of EF scores (both on the BRIEF and using laboratory measures) across the lifespan in DS and +1X, both at earlier stages in development and later in life. For the DS group, studies of EF early in development would be crucial, given that research

suggests declines in intellectual functioning prior to the age of 4 (see Carr, 2012 for a review). Understanding how EF abilities develop during this period could provide important clues to this decline. In addition, studying EF abilities in middle adulthood may provide important predictive information regarding which individuals with DS will go on to develop precocious-onset Alzheimer's disease.

In the +1X group, further research is needed to examine systematically possible changes in EF skills over time. While early prospective studies of individuals with sex chromosome aneuploidies from the 1980s set the stage for a lifespan perspective on the development of these disorders (see Bender and Berch, 1987 for a review), those studies were characterized by small sample sizes. Thus, additional research is needed that examines outcomes longitudinally with larger groups and additional outcome measures.

Author Contributions

NL contributed to study design, analyzed, and interpreted data, and wrote the manuscript; PA, EW, EA, LC, and JB contributed substantially to data acquisition and provided critical revisions of the manuscript; JG, LD, DF, and JE contributed to study design and conceptualization, interpretation of data, critically revising the manuscript.

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Building an adaptive brain across development: targets for neurorehabilitation must begin in infancy

Jamie O. Edgin[1,2]*, *Caron A. C. Clark*[1], *Esha Massand*[3] and *Annette Karmiloff-Smith*[3]

[1] Department of Psychology, University of Arizona, Tucson, AZ, USA, [2] Sonoran University Center for Excellence in Developmental Disabilities, Tucson, AZ, USA, [3] Centre for Brain and Cognitive Development, Birkbeck, University of London, London, UK

Much progress has been made toward behavioral and pharmacological intervention in intellectual disability, which was once thought too difficult to treat. Down syndrome (DS) research has shown rapid advances, and clinical trials are currently underway, with more on the horizon. Here, we review the literature on the emergent profile of cognitive development in DS, emphasizing that treatment approaches must consider how some "end state" impairments, such as language deficits, may develop from early alterations in neural systems beginning in infancy. Specifically, we highlight evidence suggesting that there are pre- and early postnatal alterations in brain structure and function in DS, resulting in disturbed network function across development. We stress that these early alterations are likely amplified by Alzheimer's disease (AD) progression and poor sleep. Focusing on three network hubs (prefrontal cortex, hippocampus, and cerebellum), we discuss how these regions may relate to evolving deficits in cognitive function in individuals with DS, and to their language profile in particular.

Keywords: Down syndrome, rehabilitation, treatment, brain development, connectivity, language, hippocampus, cerebellum

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***Correspondence:**
Jamie O. Edgin, Department of Psychology, University of Arizona, 1503 East University Blvd., Tucson, AZ 85719, USA
jedgin@email.arizona.edu

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Introduction

It was not so long ago that having a neurodevelopmental disorder like Down syndrome (DS) or Fragile × syndrome (FXS) was a recipe for cognitive difficulties often deemed insurmountable. By contrast, the past two decades have offered much promise for the development of treatments for the cognitive dysfunction faced by individuals with such intellectual disabilities. Training programs have focused on processes like attention, memory and executive control (Conners et al., 2008; Bennett et al., 2013; Kirk et al., 2015). Investigations of pharmacological therapies have targeted specific cognitive skills such as the attention profile in FXS or memory processes in DS, tested via the use of animal models (Huber et al., 2002; Braudeau et al., 2011; De la Torre et al., 2014; Deidda et al., 2015). Intervention has also focused on content domains like language, demonstrating increases in spoken vocabulary with high frequency intervention (although this increase was less than the benefit achieved in non-DS groups; Yoder et al., 2014). While these studies are newly emerging and no single intervention has fully alleviated linguistic or cognitive deficits in humans with intellectual disability, these various successes have led to an increased awareness of the potential for successful intervention in a syndrome once thought too difficult to treat. In this sense, the last 10 years of work have provided a "proof of

concept" for intellectual disability neurotherapeutics which has redirected the field, providing evidence that it may be feasible to alleviate some of the cognitive difficulties associated with syndromes such as DS, on which we focus in this article.

In the past, DS (or Trisomy 21) was often simply used as a control group for studies focused on other syndromes. With recent changes in the field, however, DS has moved into the spotlight as a model condition for exploring novel interventions and, due to initial successes, DS researchers are now actively pursuing behavioral and biomedical treatments targeting this group. However, because resources are limited, we believe that the time has come to specifically reflect on which are the most effective strategies for neurocognitive intervention in DS. In our view, to assess intervention success, the field must take a more dynamic, truly developmental approach, recognizing that the "end state" of the DS neurocognitive phenotype is *emergent* across developmental time. According to the neuroconstructivist view (Karmiloff-Smith, 1998; Mareschal et al., 2007; Karmiloff-Smith et al., 2012), cognitive-level differences in older children and adults with neurodevelopmental disorders must be traced back to their more basic precursors in infancy and early childhood. Given the dynamic interplay of cognitive systems, particularly early in development prior to specialization of function, deficits in one domain may have antecedents in several other initially interrelated domains (Karmiloff-Smith, 1998; Karmiloff-Smith et al., 2012) that are traditionally considered separate or distinct in the end-state, such as face processing, space processing, number and language. Fundamentally, this means that modifying a phenotype, such as one involving language deficits, requires an in-depth understanding of how that behavior evolved over time, pinpointing its important precursors and relations to diverse cognitive systems. Based on such evidence, intervention may require treating a different set of syndrome-specific deficits at earlier points in development (e.g., treating attention to indirectly treat language; treating saccadic eye-movement planning to indirectly treat number discrimination, etc.; see discussion in Karmiloff-Smith et al., 2012).

In our discussion of the dynamic interactions between neural systems across development, we focus on the language phenotype in DS as a clinical end-point, because language is frequently noted as the most striking deficit in this group, with sustained negative implications for quality of life and day-to-day interactions (Abbeduto et al., 2007). Language is even less developed in children with DS than would be expected given their mental age in other domains (Miller and Sedley, 1995; Boudreau and Chapman, 2000; Ypsilanti et al., 2005). Since language is impaired, a natural, direct target for intervention would be language training, since this is an area of great concern for parents or caregivers of children with DS, and a function that likely would be considered an important treatment outcome by the FDA and other public policy makers. However, we will argue that more *indirect* intervention strategies need to be developed that target the specific neural and cognitive roots of language that may be disrupted by DS very early during the pre-linguistic period.

Further, given our current knowledge of brain development in DS, we posit that interventions should aim to influence global neural organization in ways that help to normalize patterns of connectivity and establish more mature brain networks, again starting as early as possible in infancy. Rapid breakthroughs in neuroscience have emphasized the gradual specialization and refinement of neural networks and cortical hubs as the hallmark of efficient, flexible adult cognition (e.g., Buckner et al., 2009). This implies that clinical endpoints for intervention should not be limited to specialized brain regions or domain-specific systems, but instead must translate to changes at the level of network organization and efficiency. Finally, because cognition evolves across time, we emphasize that it is likely that the most effective treatments will not necessarily display their effects immediately, but only over time, perhaps even years later, as children's brains develop as a function of processing increasingly complex environments. As a consequence, we might easily be misled about a treatment's efficacy if, in order to gather metrics of success, we were to focus solely on immediate or short-term outcomes. Indeed, what we know about the long-term importance of patterns established during early brain development suggests that such early neural changes may dictate not only how cognitive function develops across childhood, but also how individuals respond to the aging process as adults. In the case of DS, that aging process often includes early onset AD (i.e., over 50% after age 50 years; Zigman and Lott, 2007). What follows, then, is that the clues to supporting healthy aging in DS in the fourth and fifth decades of life may, paradoxically, be rooted in childhood or infancy (Karmiloff-Smith, in press). Given that recent evidence has demonstrated differences in brain development associated with risk for AD (APOE e4 allele) in infants and children with DS (Dean et al., 2014), more emphasis should be placed on the life-span progression to AD in both typical and DS-associated decline, beginning in infancy.

The Neural Phenotype of Down Syndrome Starts *In Utero*

Individuals with DS present with widespread differences in brain structure and function, which manifest in altered regional specializations as well as deficits in long-range neural connectivity and integration. These differences start during fetal development and continue across the lifespan. But, to set the stage, let us first briefly examine neural development in typically developing brains.

A healthy adult brain engages in processes of both information segregation and integration, resulting in efficient, flexible, cognitive processing (Sporns et al., 2000). Developmental cognitive neuroscience studies have suggested that specialized cognitive processes, such as face processing in the fusiform gyrus, become increasingly lateralized and localized to specific neural regions with time and experience (Johnson, 2001; although see Golarai et al., 2010). Such neural specialization of function may not occur in atypically developing brains, even when they display proficiency at the behavioral level (Karmiloff-Smith, 1998; D'Souza and Karmiloff-Smith, 2011). Concurrent and interactive with progressive regional specialization in healthy brains is the development of functional networks that allow for automaticity, indexing, and sustained

or modulated patterns of neural firing. Recent investigations of functional brain networks in typically-developing children and adolescents have pointed to a developmental pattern of increasing segregation between close regions, coupled with strengthened correlations between key long-range connections (Dosenbach et al., 2007; Fair et al., 2008). This process allows for the differentiation of several networks important for cognitive processing, including task-control networks ("frontal-parietal" and "cingulo-opercular"), resting state and memory networks ("the default mode network"), and a cerebellar network that is functionally connected to the task-control networks. While much of the axonal wiring of these connections likely is established by 9 months of age in typical development, the efficient co-activation of hubs in these networks continues to develop across childhood and into adolescence, and may be related to increases in myelination and synaptic remodeling (Kelly et al., 2009; Gao et al., 2011; Uddin et al., 2011). Refinement of these cortical networks involves a series of progressive and regressive events, making the tracking of the nature of atypical brain development in relation to typical trajectories essential for interpreting differences in brain structure or functional connectivity (Karmiloff-Smith, 2010).

In this article, we discuss the development of brain networks in DS, focusing on some core regions within these networks, including the hippocampus, prefrontal cortex, and cerebellum. Alterations in the structure and function of these regions have been established from neurological data in humans with DS and in animal models of the syndrome (Baxter et al., 2000; Das and Reeves, 2011; Edgin et al., 2012; Fernandez and Reeves, 2015) as well as through neuropsychological investigations (Frith and Frith, 1974; Jarrold et al., 1999; Pennington et al., 2003; Vicari and Carlesimo, 2006). Our article highlights the cascading impacts on development that may arise from atypical processing in these hubs and their connections, and concludes that important end-state targets for intervention (i.e., language) could be affected by the very early wiring and tuning of networks comprising these processing regions.

There is also evidence that the brain of individuals with DS is already aberrant prior to birth and evolves to exhibit deficits in both information segregation and the formation of efficient local representational capacity, as well as long-range connectivity. Just prior to or after birth, there are global disruptions in neurogenesis, synaptogenesis and myelination (Schmidt-Sidor et al., 1990). Reduced cell number is evident in the hippocampus and surrounding cortical regions as early as 21 weeks gestation (Guidi et al., 2008). There are increased levels of amyloid-β deposition prior to birth that continue to burden neural development across the lifespan (Busciglio and Yankner, 1995; Bahn et al., 2002; Lott et al., 2006). Ultimately, together with the formation of neurofibrillary tangles (Murray et al., 2015), this amyloid-β burden leads progressively to the transition to an AD diagnosis by middle adulthood in a majority of individuals with DS.

Structural imaging studies in older children and young adults with DS have often shown reductions in the volumes of later-developing neural structures, including the frontal lobe, hippocampus, cingulate cortex, and cerebellum. Myelination between regions also is reduced, with poor development of the white matter pathways between the frontal cortex and posterior regions, including the parietal and temporal cortices (Powell et al., 2014). Very few functional neuroimaging studies have been conducted in DS, but the available data suggest that the brains of individuals with DS may have altered functional organization, marked by over-connectivity in local functional circuits (Anderson et al., 2013; Vega et al., 2015) and deviations in the spatial distribution of neural activation, in comparison to typical activity patterns.

The regional specialization of language has been examined in two separate studies. Losin et al. (2009) used a passive story-listening paradigm (contrasting forward and backward speech) in young adults with DS in comparison to chronologically age-matched controls. While the controls showed activation in classic receptive language areas, the group with DS activated a different pattern of regions in response to the speech, including greater activation in parietal cortex. Morever, unlike in the control group, neural activation patterns in the group with DS did not differ for the forward and backward conditions, showing not only that language was processed in different regions, but also that these regions had not become specialized for meaningful vs. non-meaningful speech. A separate study by Jacola et al. (2013) revealed that individuals with DS showed greater activation in the midline regions of the frontal and cingulate cortex when listening to stories as compared to tones, suggesting a need in the group with DS to recruit greater cognitive resources to process the story. Moreover, this altered functional organization is not limited to language, because a semantic classification task for objects also yielded a pattern of brain regions differing in their spatial distribution and extent of activation (Jacola et al., 2011). Adults with DS showed activation in the middle and dorsal frontal cortex relative to an age-matched control group.

Three published studies have examined cross-regional brain connectivity in young adults with DS and each has pointed to a pattern of over-connectivity in local networks and under-connectivity of long-range connections, particularly those involved in the dorsal executive systems (i.e., dorsal prefrontal cortex). Pujol et al. (2014) used fMRI to examine functional connectivity in 20 adults with DS compared to chronologically aged-matched controls. Based on whole-brain and seed regional connectivity analyses, this study demonstrated a greater degree of connectivity in short-range connections in individuals with DS, including those in the anterior temporal lobes and amygdala, coupled with reduced connectivity in certain long-range connections, including reductions in executive network connections between the dorsal PFC, ACC and the posterior insula, circuits that have consistently been associated with executive control processes. This reduction in long-range functional connectivity was significantly and highly correlated with communication skills as assessed by parent report on an adaptive behavior assessment (i.e., the ABAS-II). Correlations with individually administered measures of verbal and nonverbal IQ were not reported, however, so it is difficult to assess the specificity of these effects.

Anderson et al. (2013) also noted variations in functional brain connectivity in adults with DS, with their analyses indicating increased local network synchrony that was idiosyncratic and disorganized across participants with DS relative to typically-developing adolescents. Interestingly, levels of anti-correlation (i.e., when activation in one area increases, activation in another decreases) between functional neural networks were lower than in the control group, a finding that was replicated by Vega et al. (2015). Given that anti-correlation generally is used as a marker of specialization and differentiation of neural networks, these findings suggest more diffuse, less organized network connectivity in the brains of individuals with DS. As in Anderson et al. (2013), Pujol et al. (2014) also found that a subset of long-range functional connections was reduced in strength. On the basis of graph theory analysis, a method for modeling pairwise connections between regions, functional connectivity within the DS group was characterized by local, as opposed to long-range networks. Moreover, the posterior hubs in the default network were absent and the attention network was not developed in the DS group. In concert with Anderson's findings, an electroencephalography (EEG) resting state analysis (Ahmadlou et al., 2013) revealed absent small-world organization in DS in the alpha- and theta-band ranges, with networks displaying more random organization.

A study examining functional connectivity using Near Infrared Spectroscopy (fNIRS) in groups of term-age infants with DS, infants born premature (<34 weeks), and a healthy, full-term control group showed that short-range connections were strong and equally developed in the preterm and full-term infants at term, but that term-age infants with DS showed reduced connectivity in these short-range connections (Homae et al., 2010). Long-range connections were not yet fully developed in any group, in keeping with findings that long-range connections only appear around 3 months postnatal development and increase thereafter. These findings indicate that short-range networks are less co-active in DS in early infancy, which is the opposite of findings in adults, but could reflect an early developing imbalance in the refinement of these networks. In total, the data suggest that the brains of individuals with DS lack the organizational structure that allows for the efficiency and flexibility found in the typically developing adult brain, and that the evolution of these differences needs to be examined across development to understand how to support healthy brain development in this group. Specifically, individuals with DS typically have immature and disorganized networks, reflecting inadequate segregation of functional regions as well as reduced long-range communication. However, if we are to utilize findings from connectivity or task-based neuroimaging in adults to determine intervention efficacy, these outcomes must first be better understood by charting differences in brain function in DS across developmental time, beginning in infancy.

Given these patterns of altered structural and functional networks in the brain, it is no surprise that language is impaired in those with DS, because this complex set of skills requires flexible interactions across multiple neural systems as well as fine-tuned local representations. Children with DS exhibit particular difficulty with expressive vocabulary as well as with the development of morphological and syntactic complexity (Chapman, 1997; Singer Harris et al., 1997; Mervis and Robinson, 2000; Fidler, 2005). In the following sections, we discuss the language profile of DS in relation to neural systems interactions that might influence these outcomes. To exemplify the neuroconstructivist perspective (Karmiloff-Smith, 1998), we frame our discussion around three neural systems of known vulnerability in DS that usually are not considered when discussing the neural underpinnings to language: the hippocampal complex, the prefrontal "executive control" system, and the cerebellum (Nadel, 2003). Only by tracking development in infancy, in relation to these neural systems and their connections, will we gain an understanding of which treatment route(s) for enhancing language acquisition may be the most effective. We now turn to these three brain circuits and their potential role in language development and delay in DS.

The Hippocampus, Memory and Language

The hippocampus is a complex and integrative circuit with an extended developmental trajectory. In typically-developing infants, the region may have some mature structural and functional properties (i.e., intrinsic oscillations) as early as birth. Accounts of early memory formation have been documented (Mullally and Maguire, 2014), but patterns of integration of the hippocampus with other regions are still being developed across early childhood. Some researchers suggest that hippocampal structure and function continues to be modified even into adulthood (Ghetti and Bunge, 2012; Demaster and Ghetti, 2013). The hippocampus is a hub in the default mode network, a resting state network including the medial prefrontal cortex, posterior cingulate, precuneus, and the parietal cortex; this network is often associated with offline, or task-independent, processing and episodic memory (Buckner et al., 2008). Examinations of default network connectivity in typical development suggest that the hippocampus doesn't show mature activation within this network until 2 years of age, a time frame that corresponds with a number of behavioral developments in memory (Gao et al., 2009; Olson and Newcombe, 2014). At 18–24 months, children can remember the spatial-temporal context of events and show flexibility in their memory, remembering items independently from their original learning context (Bauer et al., 1998; Robinson and Pascalis, 2004). These properties are hallmarks of mature hippocampal function, as the circuit serves as a spatial and temporal index for distributed cortical representations.

The hippocampus has been the focal point for many interventions in DS, as it has been shown to be altered in pre- and post-natal human development as well as in animal models of the disorder (Nadel, 2003). It has been repeatedly posited as the primary altered brain region leading to specific neuropsychological deficits in memory and learning (Pennington et al., 2003; Lavenex et al., 2015). Based on intervention successes in animal models, including therapies modulating excitatory-inhibitory balance, many current or proposed pharmacotherapies focus on altering the function

of the hippocampus (Fernandez et al., 2007; Deidda et al., 2015). While most of the therapies targeting this region have emphasized its importance for alleviating memory deficits, there is mounting evidence regarding the additional role of the hippocampus in language learning and development, the focus of this article.

Many of the functions of hippocampus might indeed support language development. It is well-established that the hippocampus indexes arbitrary relations (e.g., association between nouns and objects) and may serve to help support these fragile associations until they can be replayed, and subsequently strengthened, over periods of sleep, a topic to which we will return in some detail (Eichenbaum et al., 1994; Paller, 1997; Mayes et al., 2007). The role of the hippocampus in learning new words, which are in most instances arbitrarily associated with their referents, has been studied extensively in typical development as well as in brain-damaged adults who had developed typically until their brain insult (Breitenstein et al., 2005; Warren and Duff, 2014). While data from patients with early focal lesions to hippocampus have suggested adequate semantic and vocabulary learning, a closer examination of their learning process has actually revealed that novel fact learning is more difficult, requiring many more repetitions than in healthy controls (Gardiner et al., 2008). What about hippocampal function in DS? Studies have suggested that individuals with DS and animal models of the condition have poor memory consolidation over long-term delays (Wishart, 1993; Smith et al., 2014). Accordingly, learning curves for vocabulary acquisition are shallow for individuals with DS, who show consistent impairments in expressive vocabulary even in comparison to other intellectual disability syndromes (Mervis and Robinson, 2000; Yoder et al., 2014).

Sleep plays a particularly important role in hippocampal memory consolidation, especially in preschool children (Hill et al., 2007; Ashworth et al., 2013). It thus is likely to affect vocabulary development, and potentially language production (Gómez and Edgin, 2015; Henderson et al., 2012). While sleep problems are common to many neurodevelopmental disorders, they are particularly pronounced in DS (Ashworth et al., 2013), with difficulties ranging from insomnias (Breslin et al., 2011), to initiating/maintaining sleep as well as excessive daytime sleepiness (Cotton and Richdale, 2006; Carter et al., 2009), to physiological problems comprising a wide spectrum of sleep-related breathing abnormalities. The reduction in sleep quality for both children and adults with DS has important implications for physical, social, and cognitive performance (Fernandez and Edgin, 2013), as well as for executive control (Chen et al., 2013). Poor sleep quality may also translate into some of the everyday difficulties experienced by individuals with DS, including daytime sleepiness, irritability, hyperactivity and impulsivity (Fallone et al., 2001).

In fact, at least 30–50% of children and adults with DS experience some form of sleep disturbance, particularly sleep fragmentation and obstructive sleep apnea, where the upper airway is obstructed during sleep, resulting in intermittent hypoxia (Owens et al., 2000; Pegg, 2006; Waldman et al., 2009; Ashworth et al., 2013). Sleep apnea is a state that limits the time spent in the deepest stages of sleep (i.e., non rapid eye-movement; non-REM periods) and a sleep state that seems to be particularly important for memory consolidation, including the integration of word knowledge. This is because it is during deep sleep that the hippocampus replays memories through a series of neurophysiological events [e.g., sharp wave ripples and associated sleep spindles, brief periods of high frequency oscillations (11–16 Hz) present in non-REM; Schabus et al., 2004]. Indeed, EEG studies of sleep in individuals with DS, in line with mouse-model studies of DS (Colas et al., 2008), demonstrate increased stage-1 sleep and reduced stage-2 non-REM sleep in this population (Miano et al., 2008). In typically developing individuals, stage 1 sleep occurs between sleep and wakefulness, and is characterized by active muscular and motor activity. Although there is a decreased awareness of sensory stimuli during this stage, individuals may not subjectively perceive this as sleep. For individuals with DS, it is this stage of sleep that is increased. On the other hand, sleep spindles, which are prominent in stage-2 sleep, are reduced from birth in DS compared to typically developing infants (Ellingson and Peters, 1980). In typically developing individuals, sleep spindles have been associated with better procedural and declarative memory, as well as the integration of new memories and existing knowledge (Tamminen et al., 2010). All in all, chronic sleep difficulties in individuals with DS are likely to have profound effects on word learning by curtailing the opportunity for neural replay that is modulated by the hippocampal system. Indeed, it has been shown that sleep disruption correlates with language development in toddlers and school-age children with DS (Breslin et al., 2014; Edgin et al., in press).

It is worth noting that changes in sleep patterns have been identified in the typically developing population some 10 or more years prior to the onset of Alzheimer's symptomatology (Landry and Liu-Ambrose, 2014; Spira et al., 2014), and recent evidence suggests bi-directional causal links between sleep disturbance and the development of Alzheimer's associated neuropathology in animal models (Tabuchi et al., 2015). Therefore, the sleep disturbances prevalent in DS, together with the over-expression of the APP gene on chromosome 21, may be mechanisms contributing to the rate of progression of AD in this population (Fernandez and Edgin, 2013). While further longitudinal studies are required in order to track more fully the progression of sleep architecture over developmental time, it is clear that the role of the hippocampus in memory and language learning, together with its disruption by sleep problems, cannot be ignored.

While the hippocampus is involved in the consolidation of vocabulary, the region also mediates representational flexibility and temporal coding that could support the on-line planning and use of language. Moreover, while it has often been maintained that H.M., the most studied adult patient with hippocampal amnesia, had preserved language function after his surgery, evidenced by a stable verbal IQ, some linguistic impairments were in fact subsequently reported, including deficits on complex

language tasks and measures of verbal fluency (Corkin, 1984). It is difficult to attribute these deficits to the hippocampus proper because of the extent of H.M.'s damage to the surrounding cortex. Further studies with patients with isolated damage to the hippocampus have also reported difficulties in creative language use and flexible discourse (reviewed in Duff and Brown-Schmidt, 2012), and studies of adult patients with aphasia have also demonstrated an important role of the intact hippocampus for language recovery after stroke (Meinzer et al., 2010). Finally, functional neuroimaging studies have shown the hippocampus to be active during implicit statistical learning in adults, a mechanism often considered fundamental for grammar learning in infants (Gómez and Gerken, 1999; Schapiro et al., 2014).

Likewise, studies conducted with children with early left hemisphere lesions have pinpointed the potential role of the hippocampus as a driver of language lateralization (Liegeois et al., 2004). Indeed, in a study by Liegeois et al. (2004) early lesions to the usual left-hemisphere language areas (e.g., Broca's area specifically) did not cause reorganization of language into the right hemisphere, but children with lesions specifically to the left hippocampus did show right localized and bilateral activation during a word generation task. Together with the adult patient data, these findings yet again highlight an important role for the hippocampus in contributing to the tuning of the neural networks for language.

Much remains to be explored about the role of the hippocampus in language function, but the above evidence certainly raises the likelihood that this brain structure contributes in some capacity to language acquisition across development and to the marked delays in language acquisition in individuals with DS. While it appears that the hippocampus is important for some higher-level aspects of language, it is unclear from current data when these links would first be established. The early developing functions of the hippocampus are rarely studied in humans, as it is hard to examine the function of this deep region in infant brains. Recent work on typical development from Gómez and Edgin (2015) and Edgin et al. (2014) has emphasized the fact that the role of the hippocampus in associative, flexible learning takes developmental time, gradually becoming strengthened over the childhood period. In line with this view, it is possible that *early* language development may be supported by extra-hippocampal mechanisms, while *later* emerging language capacities benefit from the flexibility afforded by the hippocampus. If Gómez and Edgin's hypothesis regarding the late recruitment of the hippocampus for language development is correct, then early treatments supporting these networks might not yield *immediate* positive benefits on language, but may only become evident after hippocampal structures have gained full functionality and network integration (beginning at 24 months in typical development; Gao et al., 2009). In the case of those with DS, in which hippocampal development is clearly disrupted, assessing the efficacy of hippocampal circuit intervention may only be possible at an even later stage of development.

The Prefrontal Cortex, Executive Control Networks and Language

While often considered late-developing, the networks for executive control (including prefrontal cortex, PFC) turn out to be partially active already in typically developing infants and may actually play an organizing role in cortical development (Johnson, 2012). EEG coherence studies in very young typically-developing infants have suggested that frontal activity may serve as an "organizer" of posterior activity, with frontal EEG power in infancy predicting subsequent individual performance on executive tasks at preschool age (Kraybill and Bell, 2013). In a seminal study, Dehaene-Lambertz et al. (2002) found that frontal cortex was already active in typically-developing 3-month-olds while listening to forward vs. backward speech. More recently, this French group has shown that in preterm infants, the inferior frontal cortex may assist with speech sound discrimination (i.e., phoneme and talker) prior to term age at a time when neural migration is not even fully complete (Mahmoudzadeh et al., 2013). Taken together, these findings suggest that prenatal auditory experience plays an important role in the establishment of language network architecture and that the PFC may be involved in discriminating language inputs very early in typical development.

In typically-developing children, executive control develops very rapidly during the preschool period, with more gradual improvements evident through late adolescence (Best et al., 2009; Garon et al., 2008). These advancements appear to be supported by a progressive honing of the neural circuitry underlying executive control, including the frontal-parietal, dorsal-anterior and cingulo-opercular loops. The overproduction of neural spines in the prefrontal cortex is greater than in other neural regions, while synaptic pruning in this region proceeds very slowly through childhood and adolescence, providing an extended window for experience-dependent plasticity (Ferguson and Gao, 2015). Functional neural imaging studies of typically-developing cohorts indicate that the frontal-parietal control network becomes increasingly inversely correlated with the default-mode network through the course of middle childhood, with the degree of anti-correlation predicting individual differences in general cognitive performance (Gao et al., 2009; Sherman et al., 2014). Similarly, graph theory analyses show reductions in the connections between the fronto-parietal executive network and the cingulo-opurcular salience network over the course of middle childhood, concomitant with an age-related strengthening of the connections within these networks (Dosenbach et al., 2007). These changes in functional connectivity in typical development may reflect the progressive myelination of reciprocal tracts between the prefrontal cortex and other areas of the brain, including circuits to and from the limbic regions, hippocampus and striatum (Nagy et al., 2004).

Studies of typically-developing preschool and school-age children also highlight the developmental interdependency of executive control and of a number of developmental outcomes, including language, with many of these relations likely being bidirectional in nature. Inhibitory control, for example, may provide a buffer for expressive language planning, while working

memory theoretically affords the online maintenance of language inputs for processing and integration (Barkley, 1997). Executive control also has been shown to predict the development of narrative production, suggesting that it plays a fundamental role in the organization of language outputs (Friend and Bates, 2014). Any intervention to address language delays in individuals with DS will therefore benefit from the consideration of executive control networks which, as we now illustrate, are significantly atypical in DS.

While data on patterns of frontal brain connectivity are scarce in young children with DS, there is consistent evidence from adult studies to suggest that frontal volumes are selectively reduced, and that both functional and structural connectivity between the frontal cortex and the rest of the brain is altered. Data from adults with DS suggest that the fronto-parietal control network is not as clearly differentiated from the default mode network as in healthy controls (Anderson et al., 2013; Powell et al., 2014; Vega et al., 2015). Individuals with DS also show behavioral impairments on executive tasks tapping these control networks, although the degree of impairment is variable both within the DS population and across different types of tasks and age groups. Toddlers with DS show difficulties with visual sustained attention (Brown et al., 2003), although there is some suggestion that other aspects of *early* executive control, such as saccade planning and inhibitory control, may be relative strengths at this young age (Brown et al., 2003; Karmiloff-Smith et al., 2012; Roberts and Richmond, 2014). Studies conducted with older children and adolescents have generally reported pronounced deficits in verbal short-term and working memory, coupled with relative proficiency in spatial short-term memory, but impaired spatial working memory (Jarrold et al., 1999; Lanfranchi et al., 2004; Baddeley and Jarrold, 2007; Duarte et al., 2011; Yang et al., 2014). With respect to other aspects of executive control, findings in school-age children and adolescents have been mixed; some studies have reported global difficulties across multiple executive domains relative to verbal age-matched control groups (Lanfranchi et al., 2010; Borella et al., 2013). Additionally, others have reported deficits in set-shifting and selective attention (Rowe et al., 2006; Scerif and Steele, 2011; Breckenridge et al., 2013; Carney et al., 2013), with yet others finding no executive deficits beyond what would be predicted based on general cognitive performance (Pennington et al., 2003). Importantly, there is evidence that executive difficulties in DS may increase with age, particularly of course with the onset of Alzheimer's dementia (Nelson et al., 2005; Ball et al., 2008).

The uneven profile of executive impairments in DS has clear implications for intervention and resilience, especially in the light of suggestions that executive control may help to compensate for poor functioning in other domains (Halperin and Schulz, 2006; Shaw et al., 2006; Johnson, 2012). On the one hand, in individuals with DS poor network connectivity between the PFC and other neural regions may limit the potential for plasticity and diminish the ability of prefrontal regions to coordinate and modulate sensory and semantic inputs. In particular, verbal working memory deficits in DS are likely to have cascading implications for language comprehension and everyday social interactions. Difficulties with selective attention and set-shifting may also place constraints on the amount of linguistic information that individuals with DS are able to process. On the other hand, relative strengths in at least some areas of executive control (e.g., spatial short-term memory) may help to "bootstrap" language by offering alternative processing mechanisms and management strategies. Individuals with aphasia, for example, show activation of executive control networks to a greater extent than healthy controls during normal speech (Brownsett et al., 2014), and there is evidence that executive training may facilitate recovery from aphasia (Seniów et al., 2009; Lee and Moore Sohlberg, 2013). Given that executive control appears to be particularly vulnerable to aging in DS, it may be possible to encourage the development of executive skills very early in development to mitigate later cognitive decline. Although poor executive control in DS is unlikely to be the *cause* of decreased language abilities, interventions that target improvement of executive control skills or minimize the cost of poor executive control while training language, through supports that lessen working memory and attention demands, may be more effective (see Kirk et al., 2015).

In devising a strategy for considering the role of executive control in cognitive and language development, and ways to mitigate these difficulties, it is important to consider the syndrome-specific profile of DS. First, more work is needed to understand which domains of executive control can be improved via cognitive training and which domains may show little or no improvement with training in DS. It is also likely that training strategies for a population with moderate to severe cognitive impairment will need to provide more basic scaffolding than in less severe disorders like ADHD, given that multiple cognitive systems necessary for engaging with the training are also probably impaired in DS (Kirk et al., 2015). In total, intervention strategies likely to result in more mature patterns of frontal connectivity are needed, as is a better understanding of whether or not those with DS can use the compensatory resources of the frontal cortex to their advantage. Given the consistent profile of early differences in network connectivity and decreased integration of frontal cortex with posterior regions, interventions in this domain must begin as early in development as possible, starting in infancy.

The Cerebellum and Language

There is accumulating evidence from typical development of the importance of the cerebellum for almost all aspects of cognition, including language, executive control, spatial processing, memory, and social emotional processing (O'Halloran et al., 2012; Noroozian, 2014; Highnam and Bleile, 2015). This is reflected in the detailed topography and dense feed-forward and feedback loops to and from multiple regions of the cortex via the brainstem and thalamus (Stoodley, 2012; Buckner, 2013). Resting state functional MRI studies also provide evidence for the involvement of the cerebellum in multiple functional networks, including a motor control network, a multisensory network and an executive network, with connections between the cerebellum and language regions being especially dense (Buckner, 2013; Kipping et al., 2013). Deviations or slowing in

the pace of cerebellar development, therefore, are likely to have widespread implications for diverse functions.

The cerebellum has long been recognized to be critical for the smooth execution of movement, including articulatory movements important for speech and language. It is equipped with learning mechanisms based on long-term depression that allow it to modify and adapt motor schemas as a function of external feedback (Ito, 2005; Koziol and Lutz, 2013). Although initial learning of motor sequences requires extensive involvement of cortical regions, the cerebellum becomes important for the storage of these motor schemas as they become automatic or subconscious (Ito, 2005). Given its prominent role in motor coordination, deficits in the control of articulation, gait and proprioception may well relate to cerebellar compromise in individuals with DS (Mazzone et al., 2004; Carvalho and Almeida, 2009). Even in neurodevelopmental disorders such as Williams syndrome, which presents with significantly better language production than DS, individuals experience serious problems with memory for and the timing of oro-facial articulation sequences (Krishnan et al., 2013).

It is possible that the cerebellum plays an even more important role in the acquisition of new knowledge and skills in young children, given its involvement in procedural learning (Steinlin, 2007, 2008). In healthy brains, the cerebellum increases dramatically in volume through the first year of life and shows a decrease in volume beginning in middle childhood (Knickmeyer et al., 2008; Holland et al., 2014; Wierenga et al., 2014). Recent studies suggest that cerebellar disturbances during its most rapid period of development—the prenatal period and in infancy—may be the most devastating for longer-term outcomes, with broad impacts on motor control, attention and language (Riva and Giorgi, 2000; Limperopoulos et al., 2009; Brossard-Racine et al., 2015). Lesions within the cerebellum also are related to disturbances in remote regions of cortex, emphasizing the systemic implications of cerebellar disturbances on connectivity (Limperopoulos et al., 2014). It is also worth noting that, in healthy brains, there is direct connectivity between the cerebellum and inferior colliculus that bypasses auditory cortex (Coleman and Clerici, 1987).

The cerebellum theoretically acts as a repository of procedural schemas or models for how to act on the environment, particularly with respect to timing and sequencing of activity (Stoodley, 2012; Koziol and Lutz, 2013). The region appears to play an integral role in subvocal rehearsal and is especially important when demands on timing, memory and morpho-syntactic processing increase (Ackermann et al., 2007; Mariën et al., 2014). Both imaging and lesion studies indicate that the cerebellum is involved in numerous aspects of language processing, including verbal working memory, phonological processing, semantic processing, and verbal fluency (Marvel and Desmond, 2010; van den Bosch et al., 2014; Highnam and Bleile, 2015). The cerebellum also forms part of a network of regions modulating grammar and is believed to be involved in analyzing the details of speech for regularity based on grammatical rules (Caplan and Dapretto, 2001; Mariën et al., 2014). Finally, there is evidence for a strong involvement of the cerebellum in reading, where coordination of eye and voice is crucial (Mariën et al., 2014). Difficulties in all of these areas are characteristic of the language phenotype of individuals with DS.

As in other developmental disorders, such as Autism Spectrum Disorders and Fragile × syndrome, the cerebellar system is vulnerable in individuals with DS, with cerebellar volume being reduced by almost 25% relative to healthy controls (Pinter et al., 2001; Aylward et al., 2007). Histological studies indicate that aberrations in cerebellar development are probably present before birth: the cerebellum shows reduced infolding, reduced thickness in the granule layer, dramatically reduced cell production and fewer radial glia in fetuses with DS (Guidi et al., 2011). These prenatal deviations likely reflect defects in the response of precursor cells to the Sonic Hedgehog growth factor (Roper et al., 2006). Mouse models of DS also yield fewer synapses and reduced cell density, particularly of excitatory granule cells, in the cerebellum (Moldrich et al., 2007).

What are the implications for intervention with respect to cerebellar functions? Given its particular vulnerability in early childhood, its dense interconnectivity with multiple subcortical and cortical regions as well as its unique role in the timing and modulation of speech and language, the cerebellum should form a focus of treatment and intervention efforts to address language delays in DS. Notably, cerebellar involvement in AD occurs relatively late, and it has been suggested that the automatic procedural schemas stored within the cerebellum may offer an explanation for the discrepancies in performance of habitual tasks vs. memory, planning and flexibility that accompany the onset of dementia (Ito, 2005). In DS, the picture may be different, particularly if we consider this from a developmental perspective: although the onset of AD does not relate to decreases cerebellar volume (Aylward et al., 2007), disruptions to cerebellar development *early in life* may limit the potential for compensation based on well-learned procedural memories.

Therefore, interventions that target cerebellar function during its critical period of growth in infancy may have implications for the subsequent development of important adaptive skills, including language (Brunamonti et al., 2011; Schott and Holfelder, 2015). Reversal of cerebellar pathology in infancy has been accomplished in animal models (Das et al., 2013). Through the injection of a sonic hedgehog pathway agonist (SAG 1.1) at birth in the Ts65dn DS mouse model, the cerebellum was normalized in size in adulthood, an effect that resulted in improvements in learning outcomes in the model. Much remains to be explored regarding the clinical application of this treatment in humans, but the logic underlying the animal models could provide the field with a useful mechanism to explore the cerebellum's role in the development of functional brain networks throughout the lifespan.

Conclusion

This review illustrates how having an extra chromosome 21 in DS affects multiple neural systems that are likely to play a role in sculpting a healthy, adaptive brain to enable it to develop good language skills. The majority of the findings reviewed above highlight the existence of neural differences in

DS that are already present prior to birth, together with factors like amyloid deposition and sleep disruption that progressively exacerbate the problems that individuals with DS have in compensating for these early neural differences. This leads us to the conclusion that if we are to have the greatest influence on changing neuro-cognitive outcomes, we must: (1) begin treatments as early as possible in infancy, when functional dissociations are first becoming established; (2) not necessarily train in the domain of cognitive-level deficits (e.g., language) but in their basic-level underpinnings (e.g., attention, sleep); (3) incorporate temporally distal endpoint measures (i.e., both brain and neuropsychological) that embrace the interconnected nature of cognition; (4) devote more resources to understanding early patterns of brain and behavioral development in DS and how they may drive functional outcomes; and (5) gain insight into the extent to which additional burdens from amyloid deposition and sleep disruption may keep those with DS from utilizing resources to compensate for early deficits, by targeted sleep interventions. In addition to this, we stress the fact that intervention needs to be syndrome-specific (Cornish et al., 2007, 2012), that intervention is time-dependent (Karmiloff-Smith et al., 2014; Massand and Karmiloff-Smith, 2015), and that assessing whether intervention is successful must address the question of when across developmental time intervention changes are likely to become neurally manifest.

To exemplify some of these points, we consider approaches to reading intervention in individuals with DS. From early on, children with DS display atypical trajectories in reading ability (Cardoso-Martins et al., 2009). However, visual memory and word recognition skills do not appear to be as impaired as novel-word decoding and reading comprehension, which present a particular difficulty for children with DS (Bird et al., 2000). It is of interest that this profile of reading impairment is not characteristic of developmental disorders in general but seems syndrome-specific (Steele et al., 2013). For example, Steele and collaborators found that unlike children with WS matched on mental age, the reading performance profiles of those with DS were characterized by poor phonological awareness and vocabulary but good single word reading and letter knowledge. These findings illustrate our point that it is important to consider whether the long-term benefits of treatment programs may only be observed for individuals who receive treatment that is syndrome-specific, i.e., tailored to their specific profile of strengths and weaknesses in reading ability.

Several attempts have been made to better understand which types of reading and language interventions work best and which fail for children with DS. Burgoyne et al. (2012) tested the efficacy of a reading and language treatment for 57 children with DS, which included a 40-min daily intervention targeting vocabulary, phonics and word recognition. After 20 weeks of intervention, the children with DS demonstrated improved single-word reading, letter-sound knowledge, phoneme blending and expressive vocabulary. However, the results were no longer significant at 40 weeks. Moreover, the effects did not generalize to other reading and language skills, such as expressive/receptive vocabulary, non-word reading, spelling, or expressive grammar. The findings of this study suggest that although improvements can be obtained in these interventions, these are usually short term and only observed for the skills directly taught. In other words, after most interventions, children with DS do not readily generalize their learned skills to other tasks that were not directly trained (similarly to interventions targeting memory; Conners et al., 2006). On the whole, while intervention and training studies have yielded some promising short-term results for reading and language improvements for individuals with DS, most studies have failed to find long-term effects or transfer to untrained materials.

In another example, most children with DS often first learn to read using "Look and Say" approaches, which involve learning the associations between a spoken form of a word and the whole printed word (Singh and Singh, 1986). One problem with this approach is that it does not equip the child with the skills to decode newly encountered words. As an alternative to the "Look and Say" approach, children can be taught to segment a word into sounds and blend the sounds into words ("word analysis," Department for Education and Skills., 1998). Although this type of intervention improves the ability for individuals to "sound out" words, it has not been successful at improving non-word reading tests in DS, which serve as markers for how well they will do when encountering new reading materials (Goetz et al., 2008). Jarrold and Baddeley (1997) have argued that, because the auditory memory skills required for word analysis are weak in DS, the auditory information required to sound out the words is not available long enough for individuals with DS to complete the task.

Overall, then, these studies demonstrate that several current approaches to language and reading intervention have focused on direct training of word recognition and vocabulary. As we have illustrated throughout this article, deficits in vocabulary consolidation, verbal working memory, language planning and language analysis likely reflect underlying disruption to core neural hubs that manifests in different ways across development. For instance, disruptions to sleep likely mean that vocabulary training may quickly be lost in individuals with DS because hippocampal replay is not allowing for effective consolidation of this knowledge. Sleep interventions beginning in infancy may therefore have compounding positive implications for reading and language. Difficulties in planning and verbal short-term memory may also hinder reading comprehension and limit the efficacy of sound-blending approaches to intervention. Additional training and support for frontal and cerebellar short-term memory and planning functions may therefore provide a useful compliment to training in phonemic awareness or word recognition.

The findings reviewed here further indicate that we should devote resources to therapies with the potential to normalize, *early* in the DS developmental trajectory, patterns of functional brain connectivity. In terms of candidate therapeutic approaches, we argue that the brain of individuals with DS must be helped to maintain the right balance between excitation and inhibition in order to strengthen and synchronize long–range connections as well as to hone local networks (Cline, 2005; Buzsaki, 2006). Indeed, one predominant theory of neuronal dysfunction in

DS invokes an imbalance between excitation and inhibition (Kleschevnikov et al., 2004; Fernandez and Garner, 2007), so studies modifying inhibition should also examine the extent to which such interventions drive alterations in brain connectivity across development. Treatments targeting the normalization of cerebellar function early in life are also promising and provide a tool with which to determine the influence of this structure on the development of functional networks. While researchers have explored the impacts stemming from cerebellar modification on hippocampal-dependent memory performance, the effects may actually be even broader extending, for instance, to language. Whereas this work has led to candidate mechanisms that could help to alleviate cognitive difficulties (Fernandez and Garner, 2007; Das et al., 2013), it should be noted that to date the above approaches have only been shown to be effective in animal models. Much work must be done to determine whether they will translate to humans; part of this task involves understanding the developmental time frames in which intervention could be most effective. We believe that these and other treatments, including behavioral interventions, should be executed as early as is possible in development (when deemed to be safe).

Finally, an open question is the extent to which those with DS may be able to benefit from compensatory functions afforded by executive networks and frontal cortex. While frontal cortex is often thought to enable compensation for deficits in other neural systems, it remains unclear whether those with DS will be able to benefit from the training of these processes in the same manner. Given our data showing that sleep disturbances also relate to variability in executive control, it may be the case that, without targeted sleep interventions, sleep deficits could also limit the ability of those with DS to utilize the frontal cortex to compensate for altered development in other systems.

Much remains to be explored at this time in history in which interventions for cognitive differences in DS are being implemented at a rapid pace. In our view, a lifespan perspective is critical (Edgin et al., 2012; Farran and Karmiloff-Smith, 2012), meaning that it is also crucial for the field to step back and determine in far more detail precisely how the cognitive and neural phenotypes of DS (and of other neurodevelopmental disorders) evolve, which would allow for the targeting of intervention at the neural systems level.

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frontiers in Behavioral Neuroscience

REVIEW
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Timing of therapies for Down syndrome: the sooner, the better

Fiorenza Stagni, Andrea Giacomini, Sandra Guidi, Elisabetta Ciani and Renata Bartesaghi *

Department of Biomedical and Neuromotor Sciences, University of Bologna, Bologna, Italy

Intellectual disability (ID) is the unavoidable hallmark of Down syndrome (DS), with a heavy impact on public health. Accumulating evidence shows that DS is characterized by numerous neurodevelopmental alterations among which the reduction of neurogenesis, dendritic hypotrophy and connectivity alterations appear to play a particularly prominent role. Although the mechanisms whereby gene triplication impairs brain development in DS have not been fully clarified, it is theoretically possible to correct trisomy-dependent defects with targeted pharmacotherapies. This review summarizes what we know about the effects of pharmacotherapies during different life stages in mouse models of DS. Since brain alterations in DS start to be present prenatally, the prenatal period represents an optimum window of opportunity for therapeutic interventions. Importantly, recent studies clearly show that treatment during the prenatal period can rescue overall brain development and behavior and that this effect outlasts treatment cessation. Although late therapies are unlikely to exert drastic changes in the brain, they may have an impact on the hippocampus, a brain region where neurogenesis continues throughout life. Indeed, treatment at adult life stages improves or even rescues hippocampal neurogenesis and connectivity and hippocampal-dependent learning and memory, although the duration of these effects still remains, in the majority of cases, a matter of investigation. The exciting discovery that trisomy-linked brain abnormalities can be prevented with early interventions gives us reason to believe that treatments during pregnancy may rescue brain development in fetuses with DS. For this reason we deem it extremely important to expedite the discovery of additional therapies practicable in humans in order to identify the best treatment/s in terms of efficacy and paucity of side effects. Prompt achievement of this goal is the big challenge for the scientific community of researchers interested in DS.

Keywords: Down syndrome, intellectual disability, mouse models, adult therapies, perinatal therapies

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Marie-Claude Potier, Centre National de la Recherche Scientifique, France

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Mara Dierssen, Genomic Regulation Center (PRBB), Spain
Jean Maurice Delabar, Centre National de la Recherche Scientifique, France

***Correspondence:**
Renata Bartesaghi, Department of Biomedical and Neuromotor Sciences, Physiology Building, Piazza di Porta San Donato 2, I-40126 Bologna, Italy
renata.bartesaghi@unibo.it

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Intellectual disability (ID) is the most serious problem of Down syndrome (DS), with a heavy impact on families and society. Intense efforts of scientists worldwide are currently trying to discover interventions that improve or even rescue ID in DS. The results summarized below suggest that therapy for DS may be possible and that appropriately timed therapies may have a large impact on ID. This achievement would give children with DS the opportunity of a normal and autonomous life, alleviate the psychological burden on their families and solve a public health problem. This review summarizes therapies attempted in mouse models of DS, focusing in particular on early interventions.

DS is Characterized by Brain Defects that can be Traced Back to Fetal Life Stages

IQ in people with DS usually falls in the moderately to severely retarded range (IQ = 25–55) and mental age is rarely over 8 years (See Rachidi and Lopes, 2008; Dierssen, 2012). The IQ in DS is not constant during life but decreases with age and an early deceleration occurs between the age of 6 months and 2 years, with a further decline in adolescents. Children with DS exhibit incomplete and delayed acquisition of motor, linguistic, cognitive, and adaptive functions, compared with developing children of the same mental age. The brain of a child with DS develops differently from a normal brain and attains a form that is reduced in size and altered in shape. Widespread neurogenesis impairment has been documented in fetuses with DS (Contestabile et al., 2007; Guidi et al., 2008, 2011) and in mouse models of DS (Chakrabarti et al., 2007; Bianchi et al., 2010a,b; Trazzi et al., 2011) during critical brain developmental stages and is one of the major determinants of ID in DS. Proliferation impairment is worsened by a reduction in the acquisition of a neuronal phenotype and a relative increase in astrogliogenesis. In contrast, there is an increase in the production of inhibitory neurons that causes an excitation/inhibition imbalance (Chakrabarti et al., 2010). In addition to neurogenesis impairment, the DS brain is characterized by dendritic hypotrophy, spine density reduction, and alterations in spine shape (Takashima et al., 1989; Becker et al., 1991; Belichenko et al., 2004; Benavides-Piccione et al., 2004; Guidi et al., 2013) and widespread alterations of various transmitter and receptor systems (see Bartesaghi et al., 2011). These defects, which imply altered network formation and functioning, are also important determinants of ID in DS.

The Ts65Dn Mouse: A Widely Used Model for Studying DS

Various mouse models have been created that are trisomic for different sets of genes of Hsa21. Animal models do not reproduce the human disease with all its complexities but rather model specific aspects of the disease and no perfect model of DS exists. The Ts65Dn mouse is the most studied and best characterized model of DS. It bears segmental trisomy for a distal region of Mmu16 that contains approximately 55% of Hsa21 conserved genes (Davisson et al., 1990). This model is additionally trisomic for approximately 50 genes that are non-hortologous to Hsa21 (Rueda et al., 2012). During the past 20 years, numerous studies have demonstrated common features between Ts65Dn and humans, and the Ts65Dn mouse is, at the moment, the only model of DS used in pre-clinical studies to develop therapies for DS (Gardiner, 2015). However, there are some aspects that make this model limited. (1) The Ts65Dn mouse lacks numerous Hsa21 orthologous genes and has some Mmu17 genes that are non-trisomic in humans. These genes may confound results of therapeutic interventions. (2) Since males are sterile, mice are generated from Ts65Dn dams. The trisomic condition of mothers could cause developmental problems of the pups independently from trisomy. Along the same line of reasoning, embryonic treatments may have beneficial effects on trisomic pups that are secondary to the beneficial effects on the trisomic dams. Due to these limitations, treatment on Ts65Dn mice may have an unpredictable clinical outcome. Nevertheless, the Ts65Dn mouse has allowed scientists to discover treatments that may also be beneficial in individuals with DS.

Brain Functions in DS can be Pharmacologically Improved

The mechanisms whereby gene triplication leads to brain developmental alteration and, hence, ID remain to be elucidated. Among the triplicated genes *DYRK1A, SIM2, DSCAM, GIRK2, Olig1*, and *Olig2, SYNJ1*, and *APP* are thought to be heavily involved in the DS neurological phenotype. Moreover, *APP* triplication appears to be a key factor that favors the almost unavoidable development of Alzheimer's disease in adults with DS. Ideally, identification of the molecular mechanisms underlying brain abnormalities in DS will provide a rational basis from which to devise therapies that, by targeting specific cellular pathway/s, may correct the developmental defects of the DS brain. Although the molecular mechanisms that disrupt brain development in DS have not been fully clarified so far, various therapies have been attempted during the past few years in the Ts65Dn mouse model showing that it is possible to pharmacologically improve cognitive performance and different aspects of the DS brain phenotype (**Tables 1**, **2**).

The Number of Pre-clinical Studies for DS has Progressively Increased during the Past Few Years

During the past 14 years the number of studies focusing on pharmacotherapies for DS has grown almost exponentially. The results of a Medline research [a group of keywords was: "Down syndrome AND mouse model AND (therapy OR treatment OR restoration OR rescue OR improvement)"; a second group of keywords was: "Down syndrome AND mouse model AND LTP"] are summarized in **Figure 1**. **Figure 1A** summarizes the number of articles published since 2002 up to the beginning of current year. While in the period 2002–2008 the overall number of articles was 15 (**Figure 1B**), with a mean number of two articles per year, in the period 2009–2015 the overall number of articles was 40 (**Figure 1B**), with a mean number of six articles per year. These figures are quite encouraging because they show that the relatively small community of researchers interested in DS is making increasing efforts to find treatment for DS. This gives us hope that this intense commitment will produce good results in a near future.

Numerous Therapies Have Been Attempted in Order to Improve the Phenotype of the Trisomic Brain

A number of therapies have been tested so far in mouse models of DS in order to improve the DS-linked brain phenotype. Since most of these therapies have been tested in the Ts65Dn mouse, the most popular model of DS, we will focus here mainly

TABLE 1 | Therapies administered at adult life stages in the Ts65Dn mouse model of DS.

Phenotype	Treatment	Mechanism	Age (M)	Treatment duration	Outcome	Long-term effect	References
L/M (MWM)	Donepezil (Class A)	AChE inhibitor	4	7 w	**Failed**	NA	Rueda et al., 2008a
L/M (SA)	Physostigmine (Class A)	AChE inhibitor	4 10 16	Acute Acute Acute	Rescued **Failed** **Failed**	NA NA NA	Chang and Gold, 2008
Olfactory learning	Galantamine (Class A)	AChE inhibitor	3–6	Acute	Rescued	NA	de Souza et al., 2011
L/M (NOR, TM)	Pentylentetrazole (Class A)	Antagonist of $GABA_A$ R	3–4	17 d	Rescued	**Yes (at 2 m)**	Fernandez et al., 2007
L/M (MWM)	Pentylentetrazole (Class A)	Antagonist of $GABA_A$ R	4	7 w	Rescued	NA	Rueda et al., 2008a
L/M (NOR)	Pentylentetrazole (Class A)	Antagonist of $GABA_A$ R	2–3	2 w	Rescued	**Yes (at 8 d)**	Colas et al., 2013
L/M (NOR)	Pentylentetrazole (Class A)	Antagonist of $GABA_A$ R	12–15	2 w	Rescued	**Yes (at 8 d)**	Colas et al., 2013
L/M (MWM)	RO4938581 (Class A)	$GABA_A$ α5 negative allosteric modulator	3–4	6 w	Rescued	NA	Martínez-Cué et al., 2013
L/M (NOR, MWM, CFC)	CGP55845 (Class A)	Antagonist of $GABA_B$ R	2–3	3 w	Rescued	NA	Kleschevnikov et al., 2012
L/M (MWM, CFC)	Ethosuximide (Class A)	Inhibits KCNJ6/GIRK2 channel, a $GABA_B$–coupled ion channel	4.5–5	10 w	**Failed**	NA	Vidal et al., 2012
L/M (MWM, CFC)	Gabapentin (Class A)	Modulator of GABA synthesis	4.5–5	10 w	**Failed**	NA	Vidal et al., 2012
L/M (CFC, nesting behavior)	L-DOPS (Class A)	NA pro-drug	6	Acute	Rescued	**No (at 2 w)**	Salehi et al., 2009
L/M (NOR, CFC, TM)	Xamoterol (Class A)	β1 receptor agonist	9–12	Acute	Rescued	NA	Faizi et al., 2011
L/M (NOR, SA)	Clozapine-N-oxide (agonist of hM3Dq, administered via adeno virus into Locus Coeruleus) (Class A)	DREADD design in order to stimulate NA neurons of Locus Coeruleus	14	Acute	Rescued	NA	Fortress et al., 2015
L/M (SA)	L-DOPS (Class A)	NA pro-drug	11	2 w	Rescued	NA	Fortress et al., 2015
L/M (CFC)	Memantine (Class A)	Antagonist of NMDA R	4–7	Acute	Rescued	NA	Costa et al., 2008; Ahmed et al., 2015
L/M (WRAM, NOR)	Memantine (Class A)	Antagonist of NMDA R	4	6 m	Improved	**No (at 1 w)**	Lockrow et al., 2011
L/M (MWM)	Memantine (Class A)	Antagonist of NMDA R	9	8–9 w	Rescued	NA	Rueda et al., 2010
L/M (YM)	RO25-6981 (Class A)	Antagonist of NMDA R (GluN2B)	3–6	Acute	**Failed**	NA	Hanson et al., 2013
L/M (YM, BM)	RO25-6981 (Class A)	Antagonist of NMDA R (GluN2B)	3–6	2 w	**Failed**	NA	Hanson et al., 2013
L/M (NOR, YM)	Fluoxetine (Class A)	Inhibits serotonine reuptake	> 2 m	8 w	Rescued	NA	Begenisic et al., 2014
L/M (MWM)	Fluoxetine (Class A)	Inhibits serotonine reuptake	5–7	4 w	**Failed**	NA	Heinen et al., 2012
L/M (YM, NPR, NOR)	JZL184 (Class A)	Inhibitor of monoacylglycerol lipase that increases levels of 2-arachidonoylglycerol	11	4 w	**Failed** (YM, NPR) Rescued (NOR)	NA	Lysenko et al., 2014
L/M (MWM)	NAPVSIPQ+SALLRSIPA (fragments of ADNP and ADNF) (Class B)	Neuroprotection against oxidative stress	10	9 d	Rescued	**No (at 10 d)**	Incerti et al., 2011
L/M (MWM)	Peptide six (fragment of CNTF) (Class B)	Neurotrophic factor	11–15	30 d	Improved	NA	Blanchard et al., 2011
L/M (TM)	Estrogen (Class B)	Protects basal forebrain cholinergic neurons	11–15	2 m	Improved	NA	Granholm et al., 2002

(Continued)

TABLE 1 | Continued

Phenotype	Treatment	Mechanism	Age (M)	Treatment duration	Outcome	Long-term effect	References
L/M (MWM, PM)	Melatonin (Class B)	Free radical scavenger	5–6	5 m	Improved	NA	Corrales et al., 2013
L/M (WRAM)	Vitamin E (Class B)	Antioxidant	4	4–6 m	Improved	NA	Lockrow et al., 2009
L/M (MWM)	Piracetam (Class B)	Nootropic	1.3	4 w	**Failed**	NA	Moran et al., 2002
L/M (MWM)	SGS-111 (Class B)	Analog of Piracetam. Nootropic	4–6	6 w	**Failed**	NA	Rueda et al., 2008b
L/M (WRAM)	Minocycline (Class B)	Anti-inflammatory	7	3 m	Improved	NA	Hunter et al., 2004
L/M (MWM, NOR, CFC)	Lithium (Class C)	Mood stabilizer. Interferes with GSK3β signaling	5–6	4 w	Rescued	NA	Contestabile et al., 2013
L/M (MWM)	DAPT (Class D)	Gamma-secretase inhibitor	4	Acute	Rescued	NA	Netzer et al., 2010
L/M (MWM, NOR)	Epigallocatechin-3-gallate (EGCG) (Class D)	Inhibitor of DYRK1A kinase	3	1 m	Rescued	NA	De la Torre et al., 2014
LTP	Pentylentetrazole (Class A)	$GABA_A$ R antagonist	3–4	17 d	Rescued	**Yes (at 2 m)**	Fernandez et al., 2007
LTP	RO4938581 (Class A)	$GABA_A$ α5 negative allosteric modulator	3–4	6 w	Rescued	NA	Martínez-Cué et al., 2013
LTP	CGP55845 (Class A)	Antagonist of $GABA_B$ R	2–3	3 w	Rescued	NA	Kleschevnikov et al., 2012
LTP	Picrotoxin (Class A)	Antagonist of $GABA_A$R	3–4	Acute	Rescued	Acute (slices)	Kleschevnikov et al., 2004
LTP	Picrotoxin (Class A)	Antagonist of $GABA_A$R	4–6	Acute	Rescued	Acute (slices)	Costa and Grybko, 2005
LTP	RO25-6981 (Class A)	Antagonist of NMDA R (GluN2B)	3–6	2 w	Rescued	**Yes (at 2–4.5 w)**	Hanson et al., 2013
LTP	Fluoxetine (Class A)	Inhibits serotonine reuptake	> 2	8 w	Rescued	NA	Begenisic et al., 2014
LTP	JZL184 (Class A)	Inhibitor of monoacylglycerol lipase/Endocann System	11	4 w	Improved	NA	Lysenko et al., 2014
LTP	Melatonin (Class B)	Free radical scavenger	6–6.5	5–5.5 m	Rescued	NA	Corrales et al., 2014
LTP	Lithium (Class C)	Mood stabilizer. Interferes with GSK3β signaling	5–6	4 w	Rescued	NA	Contestabile et al., 2013
LTP	Epigallocatechin-3-gallate (EGCG) (Class D)	Inhibitor of DYRK1A kinase	2–5	Acute	Rescued	Acute (slices)	Xie et al., 2008
Neurogenesis (DG)	RO4938581 (Class A)	$GABA_A$ α5 negative allosteric modulator	3–4	6 w	Rescued	NA	Martínez-Cué et al., 2013
Neurogenesis (DG)	Farmoterol (Class A)	β2 Receptor agonist	5–6	15 d	**Failed**	NA	Dang et al., 2014
Neurogenesis (DG)	Fluoxetine (Class A)	Inhibits serotonin reuptake	2–5	24 d	Rescued	NA	Clark et al., 2006
Neurogenesis (DG)	Peptide six (fragment of CNTF) (Class B)	Neurotrophic factor	11–15	30 d	Rescued	NA	Blanchard et al., 2011
Neurogenesis (DG)	Melatonin (Class B)	Free radical scavenger	6–6.5	5–5.5 m	Rescued	NA	Corrales et al., 2014
Neurogenesis (DG)	Lithium (Class C)	Mood stabilizer. Interferes with GSK3β signaling	5–6	4 w	Rescued	NA	Contestabile et al., 2013

(Continued)

TABLE 1 | Continued

Phenotype	Treatment	Mechanism	Age (M)	Treatment duration	Outcome	Long-term effect	References
Neurogenesis (SVZ)	Lithium (Class C)	Mood stabilizer. Interferes with GSK3β signaling	12	1 m	Rescued	NA	Bianchi et al., 2010a
Neurogenesis (DG)	P7C3 (Class E)	Proneurogenic drug	1–2.5	3 m	Improved	NA	Latchney et al., 2015
Dendritic hypotrophy	Farmoterol (Class A)	β2 Receptor agonist	5–6	15 d	Rescued	NA	Dang et al., 2014
Connectivity	Peptide six (fragment of CNTF) (Class B)	Neurotrophic factor	11–15	30 d	Rescued	NA	Blanchard et al., 2011
Neurodegeneration	Estrogen (Class B)	Protects basal forebrain cholinergic neurons	11–15	2 m	Rescued	NA	Granholm et al., 2002
Neurodegeneration	Estrogen (Class B)	Protects basal forebrain cholinergic neurons	9–15	2 m	Rescued	NA	Granholm et al., 2003
Neurodegeneration	Minocyclin (Class B)	Anti-inflammatory	7	3 m	Prevented	NA	Hunter et al., 2004
Neurodegeneration	Vitamin E (Class B)	Antioxidant	4	4–6 m	Prevented	NA	Lockrow et al., 2009

The classes reported in the column "Treatment" correspond to those summarized in the Section "Numerous Therapies Have Been Attempted in Order to Improve the Phenotype of the Trisomic Brain." The outcome "Rescued" means that in treated Ts65Dn mice the examined phenotype became similar to that of untreated euploid mice. The outcome "Improved" means that in Ts65Dn mice treatment ameliorated but did not rescue the examined phenotype. The text in bold in the column "Outcome" highlights treatments that were ineffective (Failed) and the text in bold in the column "Long-term effect" highlights treatments that either had (Yes) or did no had (No) a long-term effect. ADNF, Activity Dependent Neurotrophic Factor; ADNP, Activity Dependent Neuroprotective Protein; BM, Barnes Maze; CFC, Contextual Fear Conditioning; CNTF, Ciliary Neurotrophic Factor; d, day; DG, dentate gyrus; m, month; MWM, Morris Water Maze; NA, not available; NOR, Novel Object Recognition; NPR, Novel Place Recognition; PM, Plus Maze; SA, Spontaneous Alternation Task; SVZ, subventricular zone; TM, T Maze; w, week; WRAM, Water Radial Arm Maze; YM, Y Maze.

on therapies tested in this model. These therapies, which have been selected according to different rationales, can be variously classified, according to the chosen common denominators. Here we have grouped the attempted therapies into five major classes, named A–E (also reported in **Tables 1, 2**). (A) Therapies targeted to transmitter systems. (i) Therapies enhancing cholinergic trasmission in order to counteract age-related damage of the cholinergic systems; (ii) Therapies antagonizing GABAergic transmission, in order to reduce excessive inhibition; (iii) Therapies enhancing noradrenergic transmission, in order to compensate for dysfunctions of noradrenergic afferents to the hippocampus; (iv) Therapies targeted to the glutamate NMDA receptor, in order to restore its function; (v) Therapies targeted to the serotonergic system, in order to enhance defective serotonergic signaling; (vi) Therapies targeted to the endocannabinoid system, in order to increase its activity. (B) Therapies employing neuroprotective agents, antioxidants, and free radical scavengers, in order to reduce neurodegeneration, a typical feature of the DS brain. (C) Therapies targeted to perturbed signaling pathways. (D) Therapies to normalize the expression of proteins coded by triplicated genes. (E) Therapies that are known to have a proneurogenic effect.

The total number of studies for each of these five classes is shown in **Figure 2A**. It is evident that more than one half of the studies (32 out of a total of 55) that have attempted to rescue DS brain phenotypes have used drugs that act on transmitter systems. Many transmitter systems are altered in DS and by correcting altered synaptic function it may be possible to reinstate signal transfer, on one hand, and activity-dependent cellular functions, on the other. Most of the studies belonging to class A focus on the GABAergic system (**Figure 2B**). The rationale is that since an excessive inhibition characterizes the trisomic brain, it may be possible to normalize its function by reducing inhibition. The second most numerous group of therapies belongs to class B. This class may expand if we shift therapies targeted to the cholinergic system from class A to class B. The rationale for the wide use of neuroprotective agents or antioxidants depends on the fact that the trisomic brain undergoes neurodegeneration and develops an Alzheimer's-like pathology with age. Thus, neuroprotective agents may prevent or delay neurodegeneration. Of course, the classification criteria are not entirely flaw-free and categories may be overlapping. For instance, therapies acting on the cholinergic system may belong to class A of this review as well as to class B. The outcomes of therapies of the different classes can be found in **Tables 1, 2**. Note that this review reports results of pharmacological interventions that have examined one or more of these phenotypic features: learning and memory, LTP, neurogenesis/cellularity, dendritic pattern, and neurodegeneration. Therapies based on non-pharmacological approaches have not been included.

Trisomy-linked Brain Phenotypes can be Rescued by Different Therapies

By looking at **Tables 1**, **2** it appears that a variety of different agents, that act on different targets, can rescue one or more of

TABLE 2 | Therapies administered at neonatal and embryonic life stages in the Ts65Dn mouse model of DS.

Phenotype	Treatment	Mechanism	Age	Duration	Outcome	Long-term effect	References
NEONATAL TREATMENT							
L/M (CFC)	Fluoxetine (Class A)	Inhibits serotonine reuptake	P3	13 d	Rescued	**Yes (at 1 m)**	Bianchi et al., 2010a
L/M (MWM, NOR, PA)	Fluoxetine (Class A)	Inhibits serotonine reuptake	P3	13 d	Rescued	**Yes (at 3 m)**	Stagni et al., 2015
L/M (YM)	SAG (Class C)	Synthetic activator of Sonic hedgehog pathway	P0	1 injection	**Failed**	**Yes (at 4 m)**	Das et al., 2013
L/M (MWM)	SAG (Class C)	Synthetic activator of Sonic hedgehog pathway	P0	1 injection	Rescued	**Yes (at 4 m)**	Das et al., 2013
LTP (CA1)	SAG (Class C)	Synthetic activator of Sonic hedgehog pathway	P0	1 injection	Rescued	**Yes (at 4 m)**	Das et al., 2013
Cerebellar-functional deficits	SAG (Class C)	Synthetic activator of Sonic hedgehog pathway	P0	1 injection	**Failed**	**Yes (at 4 m)**	Gutierrez-Castellanos et al., 2013
Neurogenesis (DG and SVZ)	Fluoxetine (Class A)	Inhibits serotonin reuptake	P3	13 d	Rescued	**Yes (at 1 m)**	Bianchi et al., 2010a
Neurogenesis (DG and SVZ)	Fluoxetine (Class A)	Inhibits serotonin reuptake	P3	13 d	Rescued	**Yes (at 3 m)**	Stagni et al., 2015
Neurogenesis (DG)	SAG (Class C)	Synthetic activator of Sonic hedgehog pathway	P0	1 injection	**Failed**	**Yes (at 6 d)**	Das et al., 2013
Neurogenesis (Cerebellar granule cells)	SAG (Class C)	Synthetic activator of Sonic hedgehog pathway	P0	1 injection	Rescued	NA	Roper et al., 2006
Neurogenesis (DG and SVZ)	Epigallocatechin-3-gallate (Class D)	Inhibits DYRK1A kinase	P3	13 d	Rescued	NA	Stagni et al., 2014
Cellularity (DG granule cells)	Fluoxetine (Class A)	Inhibits serotonin reuptake	P3	13 d	Rescued	**Yes (at 1 m)**	Bianchi et al., 2010a
Cellularity (DG granule cells)	Fluoxetine (Class A)	Inhibits serotonin reuptake	P3	13 d	Rescued	**Yes (at 3 m)**	Stagni et al., 2015
Cellularity (Cerebellar granule cells)	SAG (Class C)	Synthetic activator of Sonic hedgehog pathway	P0	1 injection	Rescued	**Yes (at 4 m)**	Das et al., 2013
Cellularity (DG granule cells)	Epigallocatechin-3-gallate (Class D)	Inhibits DYRK1A kinase	P3	13 d	Rescued	NA	Stagni et al., 2014
Dendritic hypotrophy	Fluoxetine (Class A)	Inhibits serotonin reuptake	P3	13 d	Rescued	**Yes (at 1 m)**	Guidi et al., 2013
Dendritic hypotrophy	Fluoxetine (Class A)	Inhibits serotonin reuptake	P3	13 d	Rescued	**Yes (at 3 m)**	Stagni et al., 2015
Connectivity	Fluoxetine (Class A)	Inhibits serotonin reuptake	P3	13 d	Rescued	**Yes (at 1 m)**	Stagni et al., 2013
Connectivity	Fluoxetine (Class A)	Inhibits serotonin reuptake	P3	13 d	Rescued	**Yes (at 3 m)**	Stagni et al., 2015
PRENATAL TREATMENT							
L/M (RAWM)	Choline supplement (Class A)	Precursor of acetylcholine	Dams	E + 21 d	Improved	**Yes (at 13–17 m)**	Velazquez et al., 2013
Visual attention tasks	Choline supplement (Class A)	Precursor of acetylcholine	Dams	E + 21 d	Improved	**Yes (at 6–12 m)**	Moon et al., 2010
L/M (RAWM)	Choline supplement (Class A)	Precursor of acetylcholine	Dams	E + 21 d	Improved	**Yes (at 13–17 m)**	Ash et al., 2014
L/M (CFC)	Fluoxetine (Class A)	Inhibits serotonin reuptake	E10	Up to E20/21	Rescued	**Yes (at 1.5 m)**	Guidi et al., 2014
Motor and sensory milestones	NAPVSIPQ+SALLRSIPA (Class B)	Active fragments of ADNP and ADNF	E8	Up to E12	Rescued	**Yes (at P5–P20)**	Toso et al., 2008
L/M (MWM)	NAPVSIPQ+SALLRSIPA (Class B)	Active fragments of ADNP and ADNF	E8	Up to E12	Rescued	**Yes (at 8–10 m)**	Incerti et al., 2012

(Continued)

TABLE 2 | Continued

Phenotype	Treatment	Mechanism	Age	Duration	Outcome	Long-term effect	References
L/M (MWM)	Vitamin E (Class B)	Antioxidant	Dams	E+12 w	Improved	NA	Shichiri et al., 2011
Motor and sensory milestones L/M (MWM, PA)	SGS-111 (Class B)	Analog of Piracetam; Nootropic	Dams	E+5 m	**Failed**	NA	Rueda et al., 2008b
Neurogenesis (DG)	Choline supplement (Class A)	Precursor of acetylcholine	Dams	E + 21 d	Improved	**Yes (at 13–17 m)**	Velazquez et al., 2013
Neurogenesis (all brain regions)	Fluoxetine (Class A)	Inhibits serotonin reuptake	E10	Up to E20/21	Rescued	**Yes (at 1.5 m)**	Guidi et al., 2014
Cellularity (all brain regions)	Fluoxetine (Class A)	Inhibits serotonin reuptake	E10	Up to E20/21	Rescued	**Yes (at 1.5 m)**	Guidi et al., 2014
Cellularity (DG granule cells)	A-tochopherol (Class B)	Antioxidant	Dams	E+12 w	Rescued	NA	Shichiri et al., 2011
Dendritic hypotrophy	Fluoxetine (Class A)	Inhibits serotonin reuptake	E10	Up to E20/21	Rescued	**Yes (at 1.5 m)**	Guidi et al., 2014
Connectivity	Fluoxetine (Class A)	Inhibits serotonin reuptake	E10	Up to E20/21	Rescued	**Yes (at 1.5 m)**	Guidi et al., 2014
Neurodegeneration	Choline supplement (Class A)	Precursor of acetylcholine	Dams	E + 21 d	Improved	**Yes (at 13–17 m)**	Ash et al., 2014
Neurodegeneration	Choline supplement (Class A)	Precursor of acetylcholine	Dams	E + 21 d	Improved	**Yes (at 4.3–7.5 m)**	Kelley et al., 2014

The classes reported in the column "Treatment" correspond to those summarized in the Section "Numerous Therapies Have Been Attempted in Order to Improve the Phenotype of the Trisomic Brain." The outcome "Rescued" means that in treated Ts65Dn mice the examined phenotype became similar to that of untreated euploid mice. The outcome "Improved" means that in Ts65Dn mice treatment ameliorated but did not rescue the examined phenotype. The text in bold in the column "Outcome" highlights treatments that were ineffective (Failed) and the text in bold in the column "Long-term effect" highlights treatments that either had (Yes) or did no had (No) a long-term effect. CFC, Contextual Fear Conditioning; d, day; DG, dentate gyrus; E, embryonic; m, month; MWM, Morris Water Maze; NA, Not Available; NOR, Novel Object Recognition; PM, Plus Maze; TM, T Maze; RAWM, Radial Arm Water Maze; SVZ, subventricular zone; w, week; YM, Y Maze.

FIGURE 1 | Number of studies focused on pharmacotherapies in Down syndrome mouse models in the period 2002–2015. (A) Number or studies per year. **(B)** Cumulative number of studies in the period 2002–2008 and 2009–2015.

the DS brain phenotypes. For instance, memory can be improved by antagonizing GABA receptors (**Table 1**) or by antagonizing the NMDA receptor (**Table 1**); neurogenesis can be increased by drugs that interact with GSK3β, such as lithium (**Table 1**), or drugs that interact with the serotonergic system, such as fluoxetine (**Tables 1**, **2**). The outcomes of the studies reported in **Table 1** are summarized in **Figure 3A**. Importantly, 36 out of 58 interventions obtained the full rescue of the examined phenotype (**Figure 3A**, Rescued); 11 interventions obtained an improvement (**Figure 3A**, Improved); four interventions obtained the rescue of some of the examined phenotypes but not others (**Figure 3A**, Failed/Rescued); and only seven interventions were ineffective (**Figure 3A**, Failed). It must be observed that the studies reported in **Table 1** used mice of different ages and treatments with different durations. Thus, it cannot be ruled out that the ineffectiveness of some treatments may be related to the age of mice and/or to an insufficient treatment duration. In addition, it must be emphasized that the results of treatment ("rescue," "improvement" and "failure") reported in the column "Outcome" of **Table 1** refer to the specific phenotype indicated in the first column. We must be aware that the rescue of a given phenotypic feature may not necessarily lead to a cognitive improvement. Although we take these limitations into account, if we group together interventions that elicit a rescue or an improvement of the observed phenotype/s it ensures that 51 out of 58 interventions (88%) have a positive impact on the DS brain. We believe that this is an extremely important success that may give new hope for DS.

The question now arises as to how widely different approaches may produce the same result. It should be observed that the gene burden in DS alters numerous cellular pathways. Different signaling pathways concur, in many cases, to regulate the same cellular process. Thus, pharmacological restoration of a single pathway may be sufficient to correct a given defect. Consequently, therapies interacting with different pathways may ultimately lead to similar results. This aspect should not be disregarded, because the possibility to have a panel of effective therapies at hand will give us the opportunity to select the agent with as few side effects as possible.

Although animal models are essential for translation of drug findings from bench to bedside, we must be aware of possible

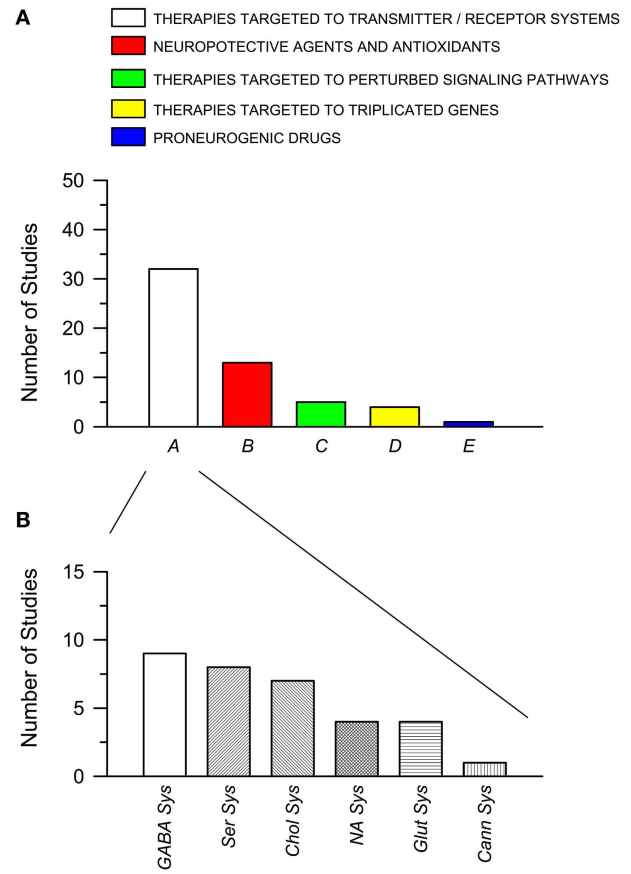

FIGURE 2 | Pharmacotherapies used in the Ts65Dn mouse models of Down syndrome grouped per class. (A) Pharmacotherapies grouped into five classes **(A–E)**, as explained in the text. The histogram shows the number of published studies for each class. **(B)** Number of studies for each subclass of pharmacotherapies belonging to class A (see text for explanation). Abbreviations: Cann Sys, cannabinoid sysem; Chol Sys, cholinegic system; GABA Sys, GABAergic system; Glut Sys, glutamatergic system; NA Sys, noradrenergic system; Ser Sys, serotonergic system.

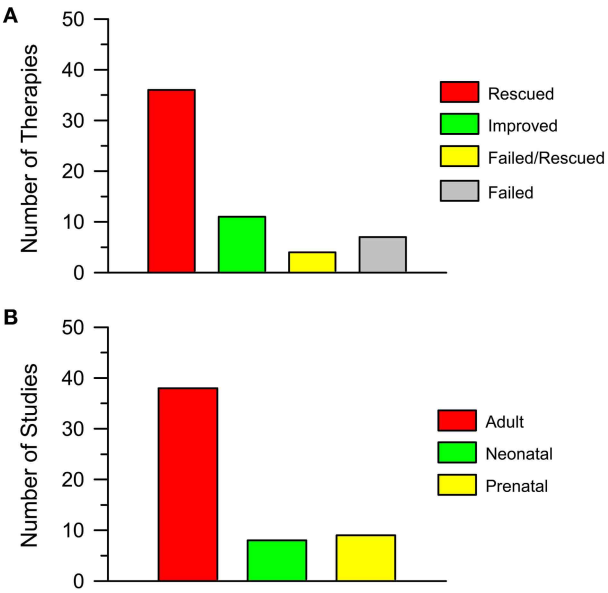

FIGURE 3 | Pharmacotherapies in the Ts65Dn mouse model of Down syndrome. (A) The histogram shows the number of attempted pharmacotherapies in the Ts65Dn mouse model of DS that rescued (Rescued), improved (Improved), partially rescued (Failed/Rescued) the examined phenotype/s or had no effect (Failed). Data derive from **Tables 1**, **2**. **(B)** The histogram shows the number of studies in which pharmacotherapies were administered at adult life stages, in the neonatal period and in the prenatal (in some instances plus post-natal) period. Data derive from **Tables 1, 2**.

limitations of the treatments attempted in mouse models in terms of translational value. The best validated animal model is not able to yield conclusive data when the experimental design is flawed or the execution of the study is not well-controlled. Yet, the studies reported in **Tables 1, 2** were (a) conducted on the Ts65Dn mouse, which in spite of some limitations replicates many aspects of the human disease, and (b) targeted to molecular alterations or phenotypic features present in the model and in the DS brain. These studies may provide, therefore, a good starting point that, after better characterization of dosing, timing, and absence of short- and long-term side effects may help in the design of future clinical trials.

Is there an Optimum Timing of Therapies for DS?

Most of the attempts to pharmacologically improve trisomy-linked brain alterations have been made in adult mice (compare **Tables 1, 2**). **Figure 3B** summarizes the number of studies in the Ts65Dn mouse models of DS that have tested the effects of pharmacotherapies at adult life stages, during the neonatal period, and during the embryonic period. Therapies were administered at adult life stages in 38 out of 55 studies (69%), in the neonatal period in eight studies (15%) and in the prenatal or prenatal + neonatal period in nine studies (16%). This striking imbalance deserves a comment. As hinted above, neurodevelopmental defects in people with DS (and mouse models of DS) are already present at fetal life stages. This is the period in which the bulk of neurogenesis takes place (**Figure 4**). There are two important exceptions to this rule: the hippocampal dentate gyrus and the cerebellum, two regions where granule neuron production largely occurs in the very early post-natal period. While in the hippocampal dentate gyrus neurogenesis goes on (at a slow rate) throughout life, in the cerebellum neurogenesis stops shortly after the early post-natal period (**Figure 4**). In view of the time course of brain development we can envisage that: (i) adult therapies may modulate ongoing hippocampal neurogenesis and, possibly, already existing hippocampal and extrahippocampal circuits. In addition, adult therapies may be used in order to prevent AD-linked neurodegeneration; (ii) neonatal therapies may largely shape hippocampal and cerebellar development; (iii) prenatal therapies may have by far the largest impact, by potentially affecting development of the whole brain (**Figure 4**). Therefore, we can expect that, while late therapies may modify the trisomic

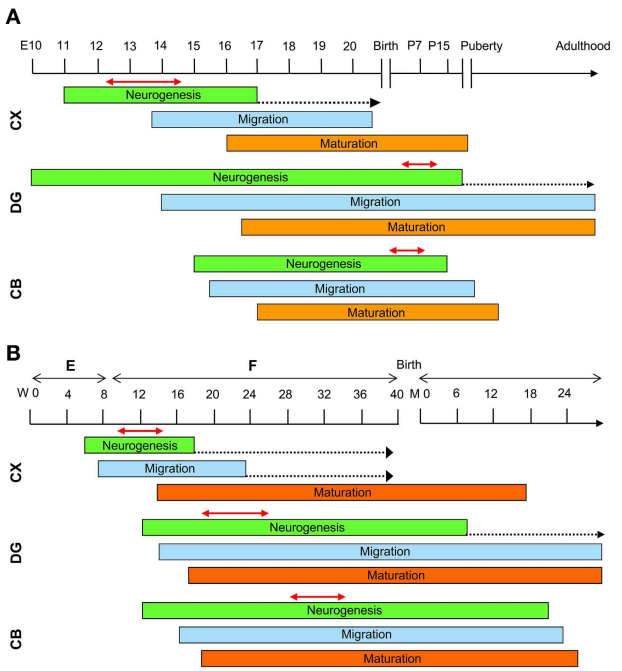

FIGURE 4 | Schematic representation of the timeline of brain development. Timeline of mouse **(A)** and human **(B)** brain development. See text for explanations. The dotted arrows indicate a reduction in the rate of neurogenesis. The double-headed red arrows delineate the period of maximum neurogenesis in the different brain regions of the mouse and human brain. Abbreviations: CB, cerebellum; CX, neocortex; DG, dentate gyrys; E, embryonic; F, fetal; M, month; P, post-natal; W, week.

brain to a relatively limited extent, perinatal therapies are likely to exert more widespread effects, potentially affecting overall brain development. In the following sections we will summarize what we currently know about the efficacy of pharmacotherapies during different life stages in the Ts65Dn mouse model of DS. However, since this review intends to focus on the impact of early therapies, the effects of therapies at later life stages will only be briefly mentioned. For more details, the reader may refer to excellent recent reviews (Costa and Scott-McKean, 2013; Gardiner, 2015).

Adult Therapies

As mentioned above, most of the studies that have sought to pharmacologically improve the DS brain phenotype have mainly used adult mice. These studies focused on the hippocampus because hippocampal-dependent learning and memory are severely affected in DS. **Table 1** summarizes the results of studies in adult mouse models of DS obtained during the past 14 years. Results of different therapies are grouped by the phenotypic features that have been examined. Since, in many instances, more than one feature has been taken into account in the same study, that study may appear more than once. The advantage of reporting results in this way is that i) the impact of different therapies on the same phenotypic feature and ii) the number of studies that have focused on that feature can be readily appreciated. Most of the studies on adult mice have examined the effect of treatment on learning and memory (L/M), without trying to find a mechanistic link between the behavioral effects and changes in the architecture and/or physiology of the hippocampal circuits. A few studies have examined, in addition to L/M, long-term potentiation (LTP) at hippocampal synapses, a form of synaptic plasticity that has been classically considered to be the electrophysiological correlate of learning and memory, although this view is becoming questionable (Abbas et al., 2015). Granule neurons of the hippocampal dentate gyrus continue to proliferate across life. The adult-produced granule neurons integrate into the hippocampal circuits and appear to play a role in memory performance (Imayoshi et al., 2008). However, relatively few studies have examined the effect of treatment on hippocampal neurogenesis. Signal processing depends on proper connectivity and thus, it is important to examine the effect of treatment on dendritic architecture and connectivity. However, there is a striking lack of information regarding this issue. A study in TgDyrk1A mice shows that EGCG restores, in addition to neurogenesis, granule cell dendritic architecture (Pons-Espinal et al., 2013) but, to our knowledge, only a single study has examined the effect of treatment on dendritic architecture in the Ts65Dn mouse (**Table 1**). The Ts65Dn mouse, similarly to individuals with DS, is bound to develop AD with age. Thus, it is of relevance to establish whether AD-like pathology can be pharmacologically improved. Accordingly, some studies have addressed this issue by specifically examining neurodegeneration (**Table 1**).

The lack of a common experimental protocol across the different research groups makes it difficult to compare the efficacy of different treatments. For instance, experiments vary for factors such as age of mice, doses, duration of treatment (acute/chronic) and experimental design. In addition, a limited number of trisomy-linked phenotypes were examined by most of these studies. Thus, the effect of treatment on the non-examined features remains to be established. Yet, by examining **Table 1**, it appears that 19 out of 36 interventions (53%) that examined L/M caused rescue of L/M, 7 (19%) caused an improvement and 10 (28%) had no effect; 10 out of 11 interventions that examined LTP caused rescue of LTP and one intervention caused an improvement; six out of eight interventions that examined neurogenesis caused restoration of neurogenesis. Thus, there is a large panel of treatments that is effective in rescuing/improving the major defects of the trisomic brain, at least in the Ts65Dn mouse model, although the clinical significance of acute treatments (see **Table 1**) remains to be established. A critical aspect that has been largely neglected is whether the effects of treatment outlast treatment cessation. Only six studies have taken this important issue into account and while two of them show that the effect of the selected therapy outlasts treatment cessation, the remaining four give disappointing results by showing that the effects disappear with time. The fact that the impact of a given therapy is ephemeral should not be disregarded, because continuous administration of drugs would be needed in order to maintain their effects, which might be impracticable.

Future Directions for Adult Therapies in DS

The studies summarized in **Table 1** are promising in that they provide proof of principle demonstration that therapies can be attempted in adults with DS in order to improve learning and memory. Importantly, some of these studies have prompted clinical trials in individuals with DS (**Table 3**). Following the "pioneer" studies carried out so far, we believe that the issue of adult therapies in mouse models of DS should be readdressed in a more systematic manner in order to obtain pre-clinical results with a translational impact. (1) Druggable candidate molecules should be chosen. (2) Dosage and duration of treatment should be carefully established in order to avoid toxic effects. (3) Treatments should be administered at different times during adulthood, in order to establish whether their effect is age-dependent. (4) The effects of treatment should be examined at both the neuroanatomical and functional level, in order to establish the mechanism/s whereby a given therapy exerts its effects. (5) Evaluation of the effects of treatment should not be confined to the hippocampus but also extend to other brain regions, because changes in the synaptic organization of other brain structures may contribute to the beneficial effect of treatment. (6) Behavioral tests should be standardized. (7) The effects of a treatment should be examined after its discontinuation, in order to establish whether it leaves an enduring trace in the brain.

Early Therapies

Investigations into early therapies for DS are much less abundant in comparison with the numerous studies regarding adult therapies (see **Figure 3B**). However, the few available studies show that perinatal therapies have impressive effects on the

TABLE 3 | Clinical trials for intellectual disability in individuals with Down syndrome.

"**A Study of RG1662 in Adults and Adolescents With Down Syndrome (CLEMATIS)**" (ClinicalTrials.gov Identifier: NCT02024789)

https://clinicaltrials.gov/ct2/show/NCT02024789

"**A Study of RG1662 in Individuals With Down Syndrome**" (ClinicalTrials.gov Identifier: NCT01436955)

https://ClinicalTrials.gov/show/NCT01436955

"**Down Syndrome Memantine Follow-up Study**" (ClinicalTrials.gov Identifier: NCT02304302)

https://clinicaltrials.gov/ct2/show/NCT02304302?cond=%22Down+Syndrome%22&rank=40

"**Efficacy and Safety of Memantine Hydrochloride in Enhancing the Cognitive Abilities of Young Adults With Down Syndrome**" (ClinicalTrials.gov Identifier: NCT01112683)

https://ClinicalTrials.gov/show/NCT01112683

"**Memantine and Down's Syndrome**" (ClinicalTrials.gov Identifier: NCT00240760)

https://ClinicalTrials.gov/show/NCT00240760

"**Down Syndrome Memantine Follow-up Study**" (ClinicalTrials.gov Identifier: NCT02304302)

https://ClinicalTrials.gov/show/NCT02304302

"**Evaluating The Safety Of Donepezil Hydrochloride (Aricept) For Up To 1 Year In The Treatment Of The Cognitive Dysfunction Exhibited By Children With Down Syndrome—Follow-Up To A 10-Week, Double-Blind, Placebo-Controlled Trial**" (ClinicalTrials.gov Identifier:NCT00675025).
https://clinicaltrials.gov/ct2/show/record/NCT00675025?term=%22down+syndrome%22+AND+%22clinical+trial%22&rank=4

"**Evaluating The Efficacy And Safety Of Donepezil Hydrochloride (Aricept) In The Treatment Of The Cognitive Dysfunction Exhibited By Children With Down Syndrome, Aged 6 To 10**" (ClinicalTrials.gov Identifier: NCT00754013)

https://ClinicalTrials.gov/show/NCT00754013

"**Evaluating The Efficacy And Safety Of Donepezil Hydrochloride (Aricept) In Treating Cognitive Dysfunction Exhibited By Children With Down Syndrome**" (ClinicalTrials.gov Identifier: NCT00570128)

https://ClinicalTrials.gov/show/NCT00570128

"**Rivastigmine Study in Adolescents With Down Syndrome (DS-Riv)**" (ClinicalTrials.gov Identifier: NCT01084135)

https://clinicaltrials.gov/ct2/show/NCT01084135?term=down+syndrome&rank=35

"**Efficacy of Rivastigmine in Patients With Down Syndrome**" (ClinicalTrials.gov Identifier: NCT00748007)

https://ClinicalTrials.gov/show/NCT00748007

"**Egcg, a dyrk1a Inhibitor as Therapeutic Tool for Reversing Cognitive Deficits in Down Syndrome Individuals**" (ClinicalTrials.gov Identifier: NCT01394796)

https://clinicaltrials.gov/ct2/show/NCT01394796

"**Normalization of dyrk1A and APP Function as an Approach to Improve Cognitive Performance and Decelerate AD Progression in DS Subjects: Epigallocatechin Gallate as Therapeutic Tool**" **(**ClinicalTrials.gov Identifier: NCT01699711)

https://ClinicalTrials.gov/show/NCT01699711

"**Vitamin E in Aging Persons With Down Syndrome**" (ClinicalTrials.gov Identifier: NCT00056329)

https://clinicaltrials.gov/ct2/show/NCT00056329

"**Multicenter Vitamin E Trial in Aging Persons With Down Syndrome**" (ClinicalTrials.gov Identifier: NCT01594346)

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The clinical trials reported investigate the efficacy of RG1662 (a $GABA_A\alpha5$ negative allosteric modulator), memantine (antagonist of the NMDA receptor), Donepezil (AChE inhibitor), Rivastigmine (AChE inhibitor), EGCG (Inhibitor of DYRK1A kinase), and Vitamin E (Antioxidant) on cognitive performance in children or adults with Down syndrome.

trisomic brain and that they can rescue numerous trisomy-linked brain alterations such as neurogenesis, brain cellularity, dendritic development, connectivity, and behavior.

Neonatal Therapies

Table 2 shows that the three treatments that have been used so far in neonate Ts65Dn mice (SAG, fluoxetine, and EGCG) have a positive impact on development of the cerebellum (SAG) and hippocampus (fluoxetine and EGCG).

SAG

The cerebellum is disproportionately small in the Ts65Dn mouse and in individuals with DS and has a reduced number of granule neurons and Purkinje cells. A first pioneer study examined the effect of SAG, a synthetic activator of the Sonic hedgehog (Shh) pathway, on cerebellar neurogenesis in newborn mice (Roper et al., 2006). In rodents, most cerebellar granule neurons are produced within the first two post-natal weeks, with a peak within the first few post-natal days (Sillitoe and Joyner, 2007; Sudarov and Joyner, 2007). Trisomic granule cell precursors show a reduced response to the Sonic hedgehog protein signal *in vitro* (Roper et al., 2006), demonstrating that this is a cell-autonomous deficit. In trisomic mice a single systemic treatment with SAG at birth was found to increase neurogenesis and restore granule cell precursor populations when mice were tested at 6 days old (Roper et al., 2006). These are the first results demonstrating that an early therapy can fully reinstate defective generation of cerebellar granule neurons. A subsequent study showed that the effect of a single neonatal injection of SAG resulted in normal cerebellar morphology in tests carried out when mice reached 4 months of age (Das et al., 2013). In contrast, 6 days after a single neonatal injection of SAG, there was no improvement in the dentate gyrus proliferation deficit in Ts65Dn mice (Das et al., 2013), suggesting that SAG may differentially affect different neural precursor cell populations. Yet, neonatal treatment with SAG restored performance in a hippocampal-dependent task (MWM) and LTP at the synapse Schaffer collaterals-CA1 when mice were 4 months old (Das et al., 2013). This evidence suggests that Shh has a role, that remains to be defined, in perinatal hippocampal development, and indicates a long-lasting effect of treatment on hippocampal function, apparently independently from neurogenesis normalization. In a more recent study, newborn mice received a single injection of SAG and were examined at 4 months of age for cerebellum-dependent learning (Gutierrez-Castellanos et al., 2013). Despite the positive impact of SAG on cerebellar neuroanatomical architecture, SAG treatment failed to rescue long-term cerebellar-based learning in mice aged 4 months. The lack of effect may be attributable to the persistence of altered granule cell electrophysiological properties and to the fact that in Ts65Dn mice there are fewer Purkinje cells, the proliferation of which cannot be affected by treatment in view of their embryonic birth date (Sillitoe and Joyner, 2007; Sudarov and Joyner, 2007).

Fluoxetine

The hippocampus of Ts65Dn mice and individuals with DS is reduced in size due to severe neurogenesis alterations and dendritic hypotrophy. The serotonergic system, which is altered in DS, plays a fundamental role in neurogenesis and dendritic development and, similarly to humans with DS, Ts65Dn mice exhibit reduced expression of the serotonin 5-HT1A receptor. Therefore, we wondered whether neonatal treatment with fluoxetine, a selective serotonin reuptake inhibitor, was able to rescue hippocampal neurodevelopmental alterations. We found that, immediately after a brief neonatal treatment (from P3 to P15) with fluoxetine, hippocampal neurogenesis, and total granule cell number were fully normalized (Bianchi et al., 2010b). Importantly, 1 month after treatment cessation, treated Ts65Dn mice exhibited fully restored granule cell number, restoration of granule cell dendritic pattern, hippocampal connectivity, signal transfer from the granule cells to CA3, and hippocampal-dependent memory function (Bianchi et al., 2010b; Guidi et al., 2013; Stagni et al., 2013). In a subsequent study we examined the effects of neonatal treatment with fluoxetine when mice reached adulthood (3 months of age) and found that in neonatally-treated Ts65Dn mice hippocampal cellularity, dendritic architecture, spine density, and memory functions were still fully rescued (Stagni et al., 2015). Moreover, we found that the increased levels of the APP-derived βCTF peptide in adult Ts65Dn mice were normalized following neonatal treatment with fluoxetine. This effect was accompanied by restoration of endosomal abnormalities, a βCTF-dependent feature of DS and AD. These results show that not only does early treatment with fluoxetine enduringly restore cognitive impairment but it may also prevent early signs of AD-like pathology.

EGCG

Among HSA21 genes known to influence brain development, *Dyrk1A* is one of the potent candidate genes closely implicated in the DS neurological phenotype. Transgenic mice that overexpress Dyrk1A exhibit brain developmental defects and behavioral alterations similar to those found in DS patients and in murine models with partial MMU16 trisomies, such as the Ts65Dn mouse, which carries extra copies of several genes, including the *Dyrk1A* gene (De la Torre et al., 2014). These observations suggest that therapeutic strategies, aimed to modulate DYRK1A activity may also have a positive effect in DS. EGCG is one of the most specific inhibitors of DYRK1A kinase activity. We are currently examining the effect of epigallocatechin-3-gallate (EGCG), the major catechin in green tea on hippocampal development. This phytochemical may have fewer side effects in comparison with SAG or fluoxetine. Our results show that neonatal treatment with EGCG fully restores hippocampal neurogenesis and cellularity (Stagni et al., 2014). The duration of these effects still remains to be elucidated.

Prenatal Therapies

Five different types of prenatal therapies have been used so far in DS mouse models, four of which have a positive effect on numerous neurodevelopmental alterations (**Table 2**).

Choline

Cholinergic neurons provide the primary source of acetylcholine, a fundamental brain neurotransmitter. A common trait of

DS and AD individuals and the Ts65Dn mouse model is the degeneration of the Basal Forebrain Cholinergic Neurons (BFCNs). This group of neurons is important for (i) explicit memory function, subserved by projections from the medial septal nucleus to the hippocampus and (ii) attention and working memory, subserved by projections from the nucleus basalis to the frontal cortex. In Ts65Dn mice degeneration of the BFCNs begins at 6 months of age, and, similarly to humans with DS and AD, continues during adulthood. Based on the unavoidable degeneration of BFCNs in these pathologies, a series of related studies (Moon et al., 2010; Velazquez et al., 2013; Ash et al., 2014; Kelley et al., 2014) considered the hypothesis that improvement of BFCNs may prevent the defects related to their degeneration. Moon et al. (2010) supplemented the diet of pregnant Ts65Dn females with high concentrations (> 4.5-fold than normal) of choline, beginning at E1 and continuing during lactation until the pups were weaned at P21. This regimen had previously been shown to have several benefits on normal rodents: (i) organizational improvement on BFC neuronal systems, ii) enduring enhancement of cognitive functions (i.e., explicit memory and attention), and iii) neuroprotection against neural insults (see Moon et al., 2010). The effect of treatment on the progeny of Ts65Dn mothers supplemented with choline was evaluated starting from when mice were 6 months of age. Behavioral testing was then continued for the following 6 months (Moon et al., 2010). In order to establish whether treatment improved cognitive performance, mice were tested with a five-choice visual discrimination task. Results showed that increasing maternal choline intake during pregnancy and lactation significantly ameliorates attentional functioning of the trisomic offspring, albeit not completely. In a subsequent work (Velazquez et al., 2013) the same schedule of treatment as in Moon et al.'s study was used, plus environmental enrichment, and mice were examined when they were 13–17 months of age. Choline supplementation was found to restore hippocampal neurogenesis (evaluated with doublecortin immunostaining) and hippocampal-dependent spatial cognition, tested with the Radial Arm Water Maze. Two subsequent studies examined the effect of the same treatment on the BFCNs in mice aged 4.3–7.5 and 13–17 months (Ash et al., 2014; Kelley et al., 2014). A reduction in the number of BFCNs was found in the medial septum of Ts65Dn mice aged 13–17 months. This defect was improved by treatment (Ash et al., 2014). These findings indicate that embryonic/early post-natal choline supplementation has effects that extend to very advanced life stages. Although the mechanisms by which prenatal/neonatal supplementation of choline reinstates hippocampal neurogenesis and functions in the Ts65Dn mouse remain to be elucidated, some theories were formulated by Moon et al. (2010) and Velazquez et al. (2013). It is possible that choline mediates these beneficial effects, altering the DNA methylation status (epigenetic effects) or regulating the production of phospholipid components of membranes. Although these theories are suggestive, we know too little about the molecular mechanism of choline in DS and further studies are needed to solve these questions.

Fluoxetine

Since serotonin is essential for neurogenesis and dendritic development (Faber and Haring, 1999; Whitaker-Azmitia, 2001), we hypothesized that treatment with fluoxetine during pregnancy could rescue most of the neurodevelopmental alterations that characterize the trisomic brain. We treated pregnant Ts65Dn females from E10 to delivery with the aim of restoring the bulk of neurogenesis. We found that untreated Ts65Dn pups exhibited a severe neurogenesis reduction and hypocellularity throughout the forebrain (subventricular zone, subgranular zone, neocortex, striatum, thalamus, hypothalamus), midbrain (mesencephalon) and hindbrain (cerebellum and pons). In Ts65Dn mice embryonically-treated with fluoxetine precursor proliferation and cellularity were fully restored in all these regions. Furthermore, embryonic treatment with fluoxetine restored the expression of the 5-HT1A receptor in the subventricular zone and hippocampal regions of Ts65Dn mice (Guidi et al., 2014). To verify whether prenatal treatment with fluoxetine had enduring effects, we examined the offspring of treated and untreated mothers when mice reached 45 days of age, i.e., at 1.5 months after treatment cessation. We found that neural precursor proliferation was still restored in the two major post-natal brain neurogenic niches (subventricular zone and subgranular zone of the dentate gyrus) (Guidi et al., 2014). In addition, in the hippocampal dentate gyrus the typical reduction in neurogenesis and the relative increase in astrogliogenesis were fully corrected indicating a long-term effect on the differentiation program. The total number of granule neurons was also still restored. Furthermore, in embryonically-treated Ts65Dn mice the dendritic development of post-natally born granule neurons was normalized with full correction of the severe dendritic hypotrophy that characterizes the trisomic condition. The counterpart of this effect was restoration of pre- and post-synaptic terminals. Finally, embryonically- treated Ts65Dn mice aged 45 days exhibited restoration of cognitive performance, indicating that the positive impact of embryonic treatment on brain development was functionally effective in adulthood.

NAP+SAL

Activity-dependent neuroprotective protein (ADNP) and activity-dependent neurotrophic factor (ADNF) are essential for brain formation (Incerti et al., 2011). The active peptide fragments of these proteins, NAPVSIPQ (NAP) and SALLRSIPA (SAL), mimic the activity of their parent proteins. These peptides have been shown to exert a protective effect against oxidative stress, the severity of traumatic head injury, stroke, and toxicity associated with the Aβ peptide, and to stabilize and repair microtubules (Gozes et al., 2005, 2008). A preliminary study showed that prenatal treatment (in the period E8–E12) with NAP+SAL prevents the delay of neurodevelopmental milestones in trisomic offspring (Toso et al., 2008). At a cellular level, prenatal NAP+SAL restore altered subunits of the NMDA receptor and $GABA_A$ receptor (Vink et al., 2009), suggesting that one mechanism by which treatment exerts its effect may be the normalization of the efficacy of excitatory and inhibitory pathways. In a subsequent study the effect of prenatal treatment

(in the period E8–E12) with NAP+SAL on learning and memory was examined when the offspring had reached 8–10 months of age (Incerti et al., 2012). Prenatally-treated Ts65Dn mice exhibited a learning curve that was similar to that of untreated euploid mice. Unfortunately, the results of the probe test are not mentioned and thus it is not possible to establish the effect of this treatment on memory. Moreover, the study did not examine the effects of treatment on neurogenesis and overall brain development. However, the results prospect the possibility of potential pregnancy interventions for DS with these peptides.

SGS-111

Neurons of DS patients exhibit a three- to four-fold increase in intracellular reactive oxygen species (ROS) due to over expression of SOD1, the gene that is responsible for the formation of the enzyme superoxide dismutase that changes oxygen free radicals into hydrogen peroxide. This oxidative stress, which damages mithocondrial membrane and lipids, occurs in DS during pre- and post-natal development and can modify critical processes of neurogenesis, differentiation, migration, and survival. Therefore, oxidative stress has been linked to the brain abnormalities observed in DS. Since oxidative stress has been reported as early as in the fetal stage, SGS-111, an analog of piracetam with neuroprotective and nootropic properties, was administered to pregnant Ts65Dn females from the day of conception, throughout pregnancy, and to their pups during the following 5 months (Rueda et al., 2008b). The behavioral characterization carried out at the end of treatment showed that chronic administration of the antioxidant SGS-111 reduced the hyperactivity shown by Ts65Dn mice but failed to improve learning and memory. The lack of effects may be due to the fact that in Ts65Dn mice the MWM task is relatively independent of the neurotoxic effect of increased oxidative stress.

Tocopherol

Another important aspect of oxidative stress found in DS brains is lipid damage caused by elevated levels of lipid peroxidation. It has been reported that the concentration of isoprostanes (a marker for lipid peroxidation) in the amniotic fluid of mothers who were pregnant with DS fetuses was nine times greater than in pregnancies involving normal fetuses, suggesting that lipid peroxidation occurs early in pregnancy (Perrone et al., 2007). Therefore, the antioxidant α-tocopherol, the most biologically active form of vitamin E, was chronically administered to pregnant Ts65Dn females from the day of conception throughout the pregnancy and to their pups until adulthood, in order to prevent the developmental consequences of elevated oxidative stress (Shichiri et al., 2011). Supplementation of α-tocopherol was found to reduce acroleine, a lipid peroxidation product, in the dentate gyrus of adult Ts65Dn mice and this effect was accompanied by an increase in granule cell density. In addition, treatment ameliorated abnormal anxiety/regardlessness in the Elevated-Plus Maze task in Ts65Dn mice, improved spatial learning, and partially improved retention memory in the MWM test. No effect of treatment on hyperactivity was found in the spontaneous motor activity test.

EGCG

Although this review is focused on therapies in the Ts65Dn mouse model, we will briefly report data obtained in the transgenic YACtg152F7 mouse, a strain that over expresses DYRK1A kinase, in view of the potential impact for DS. Transgenic YACtg152F7 mice were treated with two different polyphenol-based diets, from gestation to adulthood (Guedj et al., 2009). Chronic administration of polyphenols from green tea (that include EGCG) was found to correct, in adult transgenic mice, brain weight, and thalamus-hypothalamus volume alterations that are strongly related to *Dyrk1a* gene copy number. Moreover, this treatment restored hippocampal mRNA levels for the neurotrophic factor BDNF and its plasma membrane receptor TrkB. Consistently with the positive effect of treatment on these markers of synaptic plasticity, long-term memory, assessed using the Novel Object Recognition test, was completely restored in treated transgenic mice.

Timing is All

The studies carried out in mouse models at adult life stages show that it is possible to improve or even rescue hippocampal-dependent learning and memory, although the duration of these effects still remains a matter of investigation in the majority of cases. After the period of neuron proliferation and maturation, which takes place in the prenatal and neonatal period, there is no means to increase the number of neurons forming the brain, except—to a limited extent—for the hippocampal dentate gyrus. Thus, after the critical periods of neurogenesis and synaptogenesis the brain can undergo relatively limited plastic changes and late therapies are unlikely to exert drastic changes in the brain. Yet, although late therapies may exert a limited benefit, even a partial improvement of ID in adults with DS and/or prevention of AD development would be an extremely important achievement. Importantly, the results reviewed above clearly show that therapies administered during the early stages of brain development have an extremely pronounced effect on the trisomic brain in terms of the phenotypic features that they are able to rescue and in terms of the duration of their effects. The studies in DS mouse models provide proof of principle evidence that it might be possible to rescue brain development provided that treatments are administered during the earliest phases of brain development. The magnitude and striking persistence of the effects of neonatal and prenatal interventions emphasizes the importance of early treatment in DS.

The normal ontogeny of neural development in rodents is different from humans because rodents have considerable post-natal development and humans have considerably more prenatal maturation of their nervous systems (**Figure 4**). This aspect is fundamental to the planning of a correct pharmacological intervention during a specific phase of brain development. In mice, cortical neurogenesis takes place between embryonic days E11–E17 (Takahashi et al., 1996) (**Figure 4A**). At birth, except for a few specialized regions, including the subventricular zone/rostral migratory stream, the hippocampal dentate gyrus and the cerebellar cortex, the brain enters a state of replicative quiescence. In the hippocampal dentate gyrus,

although neurogenesis begins at E10 it exhibits its maximum rate in the first two post-natal weeks and then continues at a slow rate throughout life (Altman and Bayer, 1975, 1990a,b) (**Figure 4A**). In the mouse cerebellum, granule cell production begins at approximately E15 and is accomplished by the second post-natal week (Sillitoe and Joyner, 2007; Sudarov and Joyner, 2007) (**Figure 4A**). In the human brain, after the formation of the neural tube, (by gestational week 3), neural progenitors produce neurons that migrate from the ventricular zone, the primitive epithelial sheet of dividing neural progenitor cells, to their final destination in the regions that will form the different brain parts. In the human forebrain neocortical neurons are generated during a restricted period that begins at approximately gestational week 6 and is largely completed by week 18 (Stiles and Jernigan, 2010) (**Figure 4B**). After their final division, postmitotic neurons migrate outward from the VZ and once they have reached their target regions develop axons and dendrites and begin to form synaptic connections. Synaptic production continues during the first two post-natal years (**Figure 4B**). In the human dentate gyrus, neurogenesis begins at approximately gestational week 12 and is almost accomplished within the first post-natal year (Seress et al., 2001; Rice and Barone, 2010), although, similarly to rodents, it continues at a slow rate throughout life (Eriksson et al., 1998) (**Figure 4B**). Production of cerebellar granule cells starts at gestational week 12 (ten Donkelaar et al., 2003) and continues in the first few post-natal months (Abráham et al., 2001) (**Figure 4B**). Noninvasive prenatal testing (NIPT) for DS, using massively parallel sequencing of maternal plasma DNA, facilitates early detection of affected fetuses. As envisaged by Guedj et al., if NIPT is performed at approximately 12 weeks of pregnancy there is a potential 28-week window of opportunity in which to treat the fetus by orally administering small molecules to the mother (Guedj and Bianchi, 2013; Guedj et al., 2014). Considering the timeline of brain development, treatment during weeks 12–16 of pregnancy may have a large impact on cortical neurogenesis (**Figure 4B**). Treatments after week 16 may principally modulate cortical neuron maturation and synapse formation. Finally, treatment during late pregnancy and the first years of life may have a large impact on neurogenesis in the hippocampal dentate gyrus and cerebellum. Demonstration, obtained in mouse models, that the defects of the DS brain are reversible opens a breakthrough for the prevention of intellectual disability. The timeline of human brain development (**Figure 4B**) shows that there are windows of opportunity that can be exploited in order to pharmacologically improve (and hopefully, rescue) the neurodevelopmental alterations that characterize the DS brain.

Translational Impact of Studies in Mouse Models

The discovery that early pharmacotherapies can restore brain development in mouse models of DS raises the question of the translation of these results to human beings with DS. When designing prenatal or neonatal treatments for DS two important issues must be taken into account: the placental (and blood-brain) barrier and the possible toxicity of treatment. The drugs used so far in mice cross the placental and brain barrier but their use may pose some caveats in view of potential side effects. Pharmacological stimulation of the Shh pathway with SAG in newborn infants as a therapeutic strategy might be problematic. Since chronic Shh pathway stimulation is observed in a number of tumor types, a better understanding of the side effects of Shh treatment is required. Fluoxetine, which is an antidepressant prescribed in adults and adolescents, may be safer than SAG. Although it is in clinical trial in children as a treatment for various behavioral disturbances (Alcamí Pertejo et al., 2000; DeLong et al., 2002; Hollander et al., 2005), possible side effects in neonates cannot be ruled out. Fluoxetine use in early pregnancy has been associated with a slightly increased risk of specific cardiovascular malformations (Reefhuis et al., 2015). However, another recent study conducted on a large cohort of subjects (approximately 36,700 exposed infants and 2,200,000 unexposed infants) indicates that there is not a substantial teratogenic effect of SSRI, including fluoxetine, during the first trimester of pregnancy (Furu et al., 2015). Exposure to antidepressants (including fluoxetine) during the second and third trimester does not have substantial effects on milestones of development (Einarson et al., 2009; Pedersen et al., 2010). However, the potential risk of pre-term birth (Hayes et al., 2012) and pulmonary hypertension in the neonate (Chambers et al., 2006; Olivier et al., 2013) cannot be ruled out. It must also be observed that *in utero* exposure to serotonin reuptake inhibitors may result in a neonatal withdrawal syndrome (Moses-Kolko et al., 2005; Sanz et al., 2005). Though the withdrawal effect is generally self-limited, this aspect must be taken into account. Considering the impressive effects of fluoxetine in a mouse model of DS, the side effects of prenatal exposure to fluoxetine may be considered a relatively minor problem in the face of the possible rescue of cognitive disability. At present, there are no published data on DS babies born from mothers taking fluoxetine (or other antidepressants). A pilot feasibility trial of perinatal fluoxetine treatment at the Southwestern Medical Center of the University of Texas was approved in 2014 and its start is scheduled for 2015 (Byerly, M., Carlin, M. and Horsager-Boehrer, R., 2014. A Pilot Feasibility Trial of Prenatal and Early Post-natal Fluoxetine Treatment for Intellectual Impairments of Down Syndrome https://wwwutswmedicineorg/stories/articles/year-2015/down-syndromehtml). EGCG is a phytochemical derived from green tea extracts. The use and dosage of substances that derive from plants as natural remedies for various diseases is deeply rooted in the history of mankind. Therefore, natural substances may represent attractive tools for the therapy of various disturbances, including DS. EGCG appears to be a safe phytochemical (Vacca and Valenti, 2015) and its use has numerous beneficial health effects (Kim et al., 2014). EGCG is often classified as an antioxidant but it may function as a pro-oxidant in some cellular contexts. EGCG has many actions that do not depend on anti-oxidant mechanisms, including direct interaction with proteins and phospholipids in the plasma membrane, and regulation of signal transduction pathways and transcription factors (Kim et al., 2014). It has been shown that high doses of EGCG have hepatotoxic effects (Lambert et al.,

2009). However, the doses used in pre-clinical studies in mouse models (De la Torre et al., 2014; Stagni et al., 2014) and in the clinical trials with EGCG (reported in **Table 3**) are well below those that are known to cause adverse effects. EGCG administered to pregnant rats does not have teratogenic effects (Isbrucker et al., 2006). It is not known whether EGCG may have adverse effects during pregnancy in humans. A clinical trial for young adults with DS (De la Torre et al., 2014) shows that the positive effect of treatment with EGCG on behavior tends to disappear with time. We found that neonatal treatment with EGCG rescued hippocampal development in the Ts65Dn mouse model, similarly to that with fluoxetine. At this point it is of paramount importance to establish whether EGCG administered prenatally can rescue overall brain development, similarly to fluoxetine, and whether this effect is retained with time. If so, EGCG may be a promising treatment for the prevention of ID in DS. The neuroprotective peptides NAP and SAL can be orally administered. Moreover, NAP penetrates cells and crosses the blood-brain barrier after nasal or systemic administration. This would make treatment of individuals with DS easily feasible. These peptides do not seem to have adverse effects in animal models, and functional behavioral assays in rats show no adverse side effects with NAP concentrations that are approximately 500-fold higher than the biologically active dose (see Gozes et al., 2008). The beneficial effects of embryonic treatment on learning and memory in the Ts65Dn mouse model suggest that these peptides may be employed for prenatal treatment in DS. Choline and vitamin E are important supplements that should be taken in adequate amounts, and choline in large amounts appears to be required during pregnancy to support fetal development (Yan et al., 2013). No toxic or teratogenic effects are reported in the literature following an intake of the recommended daily range dosage of choline and vitamin E. Thus, choline and vitamin E are not likely to cause adverse effects on fetuses or babies with DS. Embryonic treatment with vitamin E improves spatial learning and delays the onset of cognitive and morphological brain abnormalities in the Ts65Dn mouse model (Shichiri et al., 2011). Although vitamin E may represent a safe and effective treatment during pregnancy, its actions appear less prominent in comparison with those of other agents. Therefore, it may be useful to combine other treatments with vitamin E in order to obtain a more significant outcome. Embryonic/early post-natal choline supplementation was found to restore behavior when mice were aged 13–17 months (Velazquez et al., 2013). Since choline is considered to be a very safe nutrient, it may be used for prenatal treatment for DS. However, further studies are needed in order to establish whether choline restores the neurodevelopmental defects of the DS brain in addition to preventing age-related cognitive deterioration. No data are available regarding potential toxic effects of SGS-111 during pregnancy, and the effects of early treatment with SGS-111 are less prominent in comparison with those of other agents.

Conclusion

The exciting discovery that the brain abnormalities of mouse models of DS can be prevented with early interventions gives us reason to believe that treatments during pregnancy may rescue brain development in fetuses with DS. Importantly, three reported cases of DS babies whose mothers took high doses of vitamin B (plus other substances) during pregnancy provide encouraging results (Baggot and Baggot, 2014) and strengthen the idea that early therapies for DS may have a very positive impact on ID. For this reason we deem it extremely important to expedite the discovery of additional therapies practicable in humans, in order to identify the best treatment/s in terms of efficacy and paucity of side effects. Prompt achievement of this goal is the big challenge for the scientific community of researchers interested in DS.

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Narrative language competence in children and adolescents with Down syndrome

Marie Moore Channell[1,2]*, *Andrea S. McDuffie*[1], *Lauren M. Bullard*[1] and *Leonard Abbeduto*[1]

[1] MIND Institute, University of California, Davis, Davis, CA, USA, [2] Department of Speech and Hearing Science, University of Illinois at Urbana–Champaign, Champaign, IL, USA

This study was designed to examine the narrative language abilities of children and adolescents with Down syndrome (DS) in comparison to same-age peers with fragile X syndrome (FXS) and younger typically developing (TD) children matched by nonverbal cognitive ability levels. Participants produced narrative retells from a wordless picture book. Narratives were analyzed at the macrostructural (i.e., their internal episodic structure) and the microstructural (i.e., rate of use of specific word categories) levels. Mean length of utterance (MLU), a microstructural metric of syntactic complexity, was used as a control variable. Participants with DS produced fewer episodic elements in their narratives (i.e., their narratives were less fully realized) than the TD participants, although MLU differences accounted for the macrostructural differences between participant groups. At the microstructural level, participants with DS displayed a lower rate of verb use than the groups with FXS and typical development, even after accounting for MLU. These findings reflect both similarities and differences between individuals with DS or FXS and contribute to our understanding of the language phenotype of DS. Implications for interventions to promote language development and academic achievement are discussed.

Keywords: Down syndrome, intellectual disability, language development, narrative language, neurodevelopmental disorders

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***Correspondence:**
Marie Moore Channell
channell@illinois.edu

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INTRODUCTION

Narrative language competence, the ability to generate or retell a personal or fictional story, is a fundamental aspect of spoken language ability. In addition to its importance in maintaining cohesive conversational interactions in social situations (McCabe and Bliss, 2003; Reed and Spicer, 2003), narrative competence plays a central role in school achievement (Dickinson and McCabe, 2001). Termed a "bridge to literacy," narrative competence scaffolds the development of both reading comprehension and writing (McCabe and Peterson, 1991). The study reported here focused on evaluating the narrative competence of individuals with Down syndrome (DS), who because of several phenotypic characteristics associated with DS, lead to the hypothesis that narrative will be especially challenging for them. We examined narrative performance in individuals with DS relative to fragile X syndrome (FXS), another neurodevelopmental disorder that causes intellectual disability and delays in spoken language, so as to evaluate the syndrome specificity of our findings in DS.

Most individuals with DS have moderate to severe intellectual disability and pervasive language impairments. Across domains of language, comprehension is generally less impaired than expression, with some aspects of comprehension (e.g., vocabulary knowledge) commensurate with levels of nonverbal cognitive ability (Abbeduto et al., 2003). Expressive language, however, is impaired relative to nonverbal cognition (Chapman and Hesketh, 2000; Abbeduto et al., 2007). Social functioning is a relative strength for individuals with DS, especially in terms of their willingness to interact with a variety of social partners (Fidler et al., 2008). Further, children with DS are often regarded by others as friendly and affectionate (Gibbs and Thorpe, 1983). Despite this sociability, however, older children and adolescents with DS exhibit problems with higher-order cognitive processing of social information (Fidler, 2006; Cebula et al., 2010) and often experience difficulties navigating interpersonal interactions (Channell et al., 2015). Limited reciprocal friendships are reported during adolescence, and meaningful employment during adulthood is often not achieved (Iarocci et al., 2008).

For individuals with DS, it is likely that narrative competence will both affect and be affected by the linguistic and social cognitive impairments associated with DS. Telling a well-developed narrative requires the coordination of a complex set of skills across multiple developmental domains. For example, children must be able to integrate and organize their everyday experiences into mental representations of events. In conversation with a listener, they must be able to hold these events in mind while using spoken language to represent temporal and causal relationships in a coherent manner (Lahey and Bloom, 1994; Berman, 1995). Furthermore, narrative requires perspective taking and inferences about the mental states (e.g., emotions, plans, and goals) of story characters as well as predictions about character actions and reactions (Trabasso and Nickels, 1992). Thus, narrative language provides a window into children's development across the cognitive, linguistic, and social pragmatic domains (Hemphill et al., 1991; Berman and Slobin, 1994; Johnels et al., 2013). The present study was designed to identify areas of relative strength and weakness in the narrative skills of individuals with DS, thereby informing treatments to enhance narrative as well as spoken language competence more broadly. Such treatments also could be useful in promoting academic and social success in this population.

We examined the narrative language samples of individuals with DS at both the macrostructural and microstructural levels of analysis. Narrative macrostructure involves evaluating the events expressed in children's stories and the overall sequential organization of these story components (Ukrainetz et al., 2005). Narrative microstructure involves evaluating the lexical and grammatical structures that children use to convey story content (Justice et al., 2006).

Narrative Macrostructure in DS

Only a few studies have examined macrostructural narrative skills in individuals with DS (Boudreau and Chapman, 2000; Miles and Chapman, 2002; Kay-Raining Bird et al., 2008; Finestack et al., 2012; Hogan-Brown et al., 2013), and these studies have yielded inconsistent results. These inconsistencies may reflect differences in the experimental contexts used to elicit the narratives (e.g., wordless picture books, silent films, or single or multi-scene pictures), whether picture support was available to participants during the narrative retell, and the comparison group(s) to which participants were matched. For example, when matched by nonverbal mental age, participants with DS are likely to display relative impairments in expressive language, making it important to account for expressive syntax in addition to nonverbal cognition.

Accordingly, Boudreau and Chapman (2000) evaluated event structure (defined as the mention of key plot line components) in individuals with DS who were matched to typically developing (TD) children using either nonverbal mental age, syntax comprehension, or expressive syntax [i.e., mean length of utterance (MLU)]. When asked to recall the story presented in a silent film, participants with DS conveyed more story events in their narratives than TD participants matched by MLU but not those matched by nonverbal mental age or syntax comprehension. These findings support the premise that narrative language competence in DS is dependent upon both an understanding of story content and the ability to formulate sentences to express story meaning.

Similarly, Miles and Chapman (2002), using a participant sample overlapping with that of Boudreau and Chapman (2000), showed participants a wordless picture book (*Frog, Where Are You?*) from the series written by Mercer Mayer that have been adopted for collecting narrative language samples due to their detailed illustrations, clear event structure, and character reactions. While viewing the book, the participants with DS mentioned more plot line components and search theme elements in their narratives than a TD comparison group matched by MLU, but fewer search theme elements than a TD group matched by nonverbal mental age. Thus, individuals with DS express a higher level of conceptual knowledge in their narratives than would be expected based on their expressive language levels, but not necessarily based on their nonverbal cognitive ability.

More recently, Hogan-Brown et al. (2013) analyzed narrative story structure in individuals with autism, FXS, DS, or TD, all matched on a receptive/expressive vocabulary composite. Participants were shown a storybook (*A Bed Full of Cats*, adapted into a wordless picture book) and retold the story to an examiner while viewing the pages a second time. The authors found no significant group differences in a composite score that included the number of episodes mentioned, the number of references to the story theme, and mention of a resolution. This null finding, however, is difficult to interpret because the receptive/expressive language composite matching criterion potentially conflated differences that may have varied systematically by participant group. That is, individuals with DS, FXS, and autism display unique profiles of strength and weakness in components of receptive relative to expressive language; matching based on a composite may have overshadowed some of the more subtle, yet meaningful, between-syndrome differences and made it difficult to interpret

the relative role of either component in aspects of their narrative macrostructure.

Finally, Finestack et al. (2012) used the Narrative Scoring Scheme (Heilmann et al., 2010) to broadly evaluate narrative macrostructure (including the use of Introductions, Conflict/Resolution, and Cohesion of events) in verbally fluent adolescents and young adults with DS or FXS and younger TD children. Participants viewed another Mercer Mayer wordless picture book, *Frog Goes to Dinner*, and retold the story to an examiner while viewing the book pages a second time. When matched by nonverbal mental age, the participants with DS outperformed the TD participants and performed similarly to the participants with FXS; however, when matched by MLU, there was not a group difference in overall scores. These findings suggest that, despite lower levels of expressive language (i.e., MLU), exposure to a greater variety of life events (as reflected by their older chronological ages) may have helped the participants with DS or FXS convey their stories in a more sophisticated manner relative to the younger TD children. The authors noted, however, that the criterion of an MLU of at least 3.0 morphemes resulted in a restricted sample of participants with DS and may not reflect the heterogeneity observed in this population. Also, the holistic metric of narrative macrostructure, rather than one based on frequency of occurrence of specific narrative elements, may not have been nuanced enough to capture more subtle aspects of narrative that could further differentiate the participant groups.

In sum, the prior studies of narrative macrostructure in DS have utilized global approaches to evaluating the sequential organization of participant narratives. More global themes, however, are comprised of sequentially organized lower-order units called episodes, which, in turn, have their own internal organization. An alternative approach to measuring story organization could examine the mention of key elements organized *within* multiple episodes of a story. To this end, we developed an episode-based coding scheme to examine narrative macrostructure from stories produced in response to the wordless picture books *Frog Goes to Dinner* and *Frog on His Own*.

Narrative Microstructure in DS

Some studies have taken a microstructural approach to analyzing narrative language in individuals with DS by evaluating the linguistic structures used to communicate their narratives, focusing on MLU (expressive syntax) as well as sentence complexity. For example, Hesketh and Chapman (1998) found that children with DS produced significantly fewer grammatical verbs (e.g., forms of *do*, *be*, or *have*) and main verbs per utterance relative to TD children matched by MLU. Further, participants with DS who had MLUs in excess of 3.5 words produced a significantly higher number of different main, but not grammatical, verbs. These results suggest that MLU may play a different role in verb use in DS than in typical development. However, a comparison group of individuals with intellectual disability of another origin (e.g., FXS) is needed to determine the syndrome specificity of this finding.

Evaluating other microstructural components, Chapman et al. (1998) found that children, adolescents, and young adults with DS omitted more words than TD children matched by nonverbal mental age. Most of the words omitted by participants with DS were function words that contributed to the syntactic complexity of sentences (e.g., verb forms, articles, prepositions, pronouns, adverbs, and conjunctions). More recently, however, Thordardottir et al. (2012) found that narratives produced by older children and adolescents with DS (a subset of the sample reported by Chapman et al., 1998) did not show differences in measures of sentence complexity compared to younger TD children matched by MLU. The authors did, however, note particularly wide variability in performance within the group with DS.

In all of the microstructure-focused studies to date, narratives were collected in unstructured conversations, with variability in narrative contexts across participants even within a single study. In the Hesketh and Chapman (1998) study, for example, about two-thirds of participant narratives came from talking about a favorite book or activity, whereas the remainder consisted of retellings of a wordless picture book. Such variability makes interpretation of the findings more difficult, as these types of task differences are likely to be confounded with participant group. Moreover, the skills needed for conveying a personal narrative are likely to differ from those needed to retell a fictional story, especially if the story-telling context includes picture supports in the form of illustrations from the book.

In the current study, we analyzed aspects of narrative microstructure in youth with DS in the context of narration of a wordless picture book. We focused our microstructural analysis specifically on the use of verbs, conjunctions, and adverbs because these word classes have particular relevance to the ability to communicate event sequences that tie together the story grammar elements considered in our macrostructural analyses. The existing literature also does not inform us as to whether observed patterns of narrative microstructure are specific to the language phenotype of DS or more common to intellectual disability in general, and the relative role of MLU is still unclear. Thus, in the current study, we compared narrative microstructure in youth with DS to youth with FXS as well as younger TD children of similar nonverbal cognitive ability levels and statistically evaluated the role of MLU in the analyses.

Current Study Aims

We used both macrostructural and microstructural approaches to evaluate the narratives produced by children and adolescents with DS in response to wordless picture books. Specifically, we addressed the following research questions. (1) Is there a strength or weakness at the macrostructural level in story grammar organization in youth with DS relative to youth with FXS or TD children of similar nonverbal cognitive ability level? (2) What is the relative role of MLU (i.e., expressive syntax) in story grammar organization in youth with DS relative to the comparison groups? (3) At the microstructural level, do youth with DS differentially use grammatical word categories in their narratives relative to

youth with FXS or TD children? (4) What is the relative role of MLU in the use of different word categories in youth with DS relative to the comparison groups?

These data will contribute to ongoing efforts to further characterize the DS phenotype by identifying areas of relative strength and difficulty in spoken language use. Ultimately, greater specification of narrative language development in this population should lead to the development of more effectively targeted treatments.

MATERIALS AND METHODS

Participants

Participants were drawn from a larger study on language development in neurodevelopmental disorders and overlap with those described in previous studies (e.g., Kover et al., 2012; Finestack et al., 2013); however, all of the analyses reported in this paper have not been previously conducted or reported. In the larger study, inclusion criteria were parent report that the child used speech as a primary mode of communication, was a native English speaker, could produce at least three-word phrases in everyday speech, functioned generally at the kindergarten level or higher, and had no major uncorrected physical or sensory impairments that would interfere with the ability to perform in the project. Additionally, all participants were required to pass a hearing screening indicating a pure-tone threshold of <30 dB in at least one ear. For the present study, we also required that each participant have complete data on the Narrative Task, defined as story-relevant speech on at least 75% of the book page spreads. Seven individuals with DS did not meet this criterion due to non-compliance/lack of task completion and thus were excluded from the present study.

Participants with DS were matched to a sample of youth with FXS ($t_{(43)} = -0.332$, $p = 0.742$) and a sample of TD children ($t_{(44)} = -0.058$, $p = 0.954$) who were selected on the basis of nonverbal cognitive ability level (i.e., Leiter-R growth score values; see **Table 1**). The sample with FXS and the TD sample were also matched to each other on nonverbal cognitive ability level ($t_{(43)} = 0.274$, $p = 0.785$). All participants with FXS or TD who were selected into the comparison samples also met the present study's inclusion criteria listed above. This resulted in a final sample of 23 youth with DS (10–16 years old; 13 males, 10 females), 22 youth with FXS (10–16 years old; 19 males, 3 females), and 23 TD children (3–6 years old; 14 males, 9 females).

TABLE 1 | Descriptive characteristics by participant group.

	DS *Mean (SD) Range*	FXS *Mean (SD) Range*	TD *Mean (SD) Range*
Chronological age	12.80 (1.59) 10.28–15.54	12.33 (1.74) 10.18–16.01	4.48 (0.86) 3.11–6.19
Leiter-R growth scores	462.09 (7.66) 442–474	462.82 (7.09) 446–476	462.22 (7.58) 442–474
Leiter-R standard scores[a]	42.48 (7.07) 36–65	44.41 (7.87) 36–65	110.96 (15.50) 87–159
MLU in morphemes	5.07 (2.00) 1.40–9.17	5.11 (1.42) 2.83–7.37	6.19 (1.32) 4.07–8.83
TROG-2 raw scores	24.00 (9.34) 5–42	40.91 (17.62) 11–65	25.50 (13.41) 7–64

[a]*DS: n = 7, FXS: n = 5, TD: n = 0 scored at the floor standard score of 36.*

For participants with DS, we relied on parent report or, when available, a copy of a karyotype or physician report of a diagnosis of DS. Documentation was not available for five participants, but the remainder was documented as Trisomy 21. For participants with FXS, we required written documentation of an FMR1 full mutation based on molecular genetic testing. For the TD participants, we required that they had no diagnosis of a developmental disability and were not receiving special education services. Additionally, none of the TD participants were receiving speech/language therapy. Participants were recruited through a university registry, postings on websites and listservs, newspaper ads, and in the case of the TD children, local preschools. Approval for human subjects research was granted by the affiliated universities' institutional review boards, and written consent was obtained from parents/guardians of all participants.

Measures

Narrative Task

Participants were shown one of two wordless picture books, *Frog Goes to Dinner* (Mayer, 1974) or *Frog on His Own* (Mayer, 1973), and then told the story to an examiner while viewing the book a second time. The book version was counterbalanced across participants in the larger study. For the initial viewing of the book, each participant was told to look at the pictures so s/he could see what happened in the story, and the examiner turned the pages of the book so that each page spread was viewed for approximately 10 s. For the retell, the participant was instructed to tell the examiner everything about the story, page by page. Examiner prompts were scripted to minimize examiner scaffolding of the narrative retell. The examiner controlled the page turns and waited 5–7 s after each participant response prior to turning to the next page. Participants' narratives were audio-recorded for later transcription. The examiner said "next page" at each page turn so that transcribers were aware of the location in the book during transcription.

Transcription of Participant Narratives

Trained personnel transcribed audio files of participants' narratives verbatim using Systematic Analysis of Language Transcription (SALT; Miller and Iglesias, 2006) software. For each narrative language sample, a primary transcriber completed a first draft and then a second transcriber listened to the language sample, checked the transcription draft, and provided feedback to the primary transcriber, who was responsible for finalizing the transcript. Transcribers were highly trained according to the procedures described by Abbeduto et al. (1995). Transcribers segmented participants' speech into communication units (C-units), defined as an independent clause and its modifiers, which can include dependent clauses (Loban, 1976). Segmentation into C-units provides a more accurate measure of language ability than segmentation into

utterances for children beyond the developmental age of 3 years (Abbeduto et al., 1995). As reported by Kover et al. (2012) and Finestack et al. (2013), approximately 20% of narrative transcripts from each participant group was checked for inter-transcriber agreement, averaging 94% for TD participants, 90% for participants with FXS, and 86% for participants with DS [averaging over C-unit segmentation, intelligibility, mazes, overlaps, pauses, abandonment, word identification, number of morphemes and words, and ending punctuation (e.g., question intonation)].

Coding Story Grammar Use

Following transcription, trained study personnel coded narrative transcripts for the presence of pre-defined story grammar elements within episodes of *Frog Goes to Dinner* or *Frog on His Own*, using the coding scheme described below. We applied the story grammar paradigm set forth by Stein and Glenn (1975) to structure our coding scheme. For each book, we identified one setting at the beginning of the story, which established the location and/or timeframe of the entire story. Then, we divided each book into five episodes. For each episode, we identified the following six story grammar elements: an *Initiating Event* that triggers the episode, a character's *Internal Response* to the initiating event, the character's *Plan* or goal in response to the initiating event, the character's *Attempt* to put the plan into action, an *Outcome* or consequence resulting from the character's action, and a character's physical or psychological *Reaction* to the outcome (see **Table 2**). To guide our interpretation of the story elements in the episodes, we tallied the components of each story mentioned by a group of older TD children whose narratives were considered a "gold standard" child sample. This approach, along with reviewing the story scripts provided by SALT, provided the framework for developing the final episodic structure of each story.

Using this coding scheme, trained personnel reviewed each participant's transcript and independently identified the story grammar elements that were included in the narrative for each episode. Coders were blind to the participant's diagnostic group. Coders used a copy of the book as a reference, which, along with the page numbers provided on the transcripts, allowed them to confirm the pages that were being referenced by the participant during any part of the narrative. Coders awarded credit for any given element if the child provided enough story content on the appropriate page of the book to allow the coder to identify which element s/he was referencing. Although abandoned C-units were excluded, C-units with unintelligible segments were considered if there was enough information in the C-unit to determine its meaning. A child did not need to mention one element in order to receive credit for the next. Credit was only awarded once for each story element within an episode. If a child used more than one C-unit to relay a story element, those C-units could be considered together, but s/he still only received one point for that element. Additionally, in some episodes there were numerous ways in which a child could receive credit for a given story element. If the child correctly referenced more than one example, again s/he only received one point for the element. Thus, one initial setting plus six elements within each of five episodes resulted in a maximum story grammar score of 31 points for either book.

Approximately 20% of the narrative transcripts from each book were coded independently by a second coder to assess reliability. Point-by-point inter-coder agreement averaged 93% (range = 85–100%) for *Frog Goes to Dinner* and 96% (range = 90–100%) for *Frog on His Own*. See **Table 3** for inter-coder agreement by story grammar element type.

TABLE 2 | Story grammar elements.

	Definition	Example[a]
Initiating event	Event or problem that kicks off the episode	Frog jumps in (or is in) the saxophone
Internal response	Reference to character's psychological state in response to the initiating event	Musician wonders (or doesn't know) what happened
Plan	Reference to character's intent to act upon or resolve the problem caused by the initiating event	Musician wants to figure out why his saxophone won't play
Attempt	Character action directed at carrying out the plan	Musician tips over saxophone to look inside
Outcome	Consequence of the attempt (may not resolve the problem)	Frog lands/is on the musician's face
Reaction	Character reaction to the outcome (emotions or actions)	Musician falls into the drum/The drummer is angry

[a]*Example from Episode 2 of Frog Goes to Dinner.*

Coding Grammatical Word Category Use

We coded transcripts from participants' narratives for the use of main verbs, adverbs, and conjunctions. Semantic context was taken into consideration such that a participant did not receive credit for a word used in a nonsensical or non story-related semantic context. However, a participant received credit for a semantically appropriate use of a word even if the C-unit was not syntactically correct (e.g., "frog jump inside" would receive credit for the verb *jump*). Although abandoned C-units were excluded, C-units with unintelligible segments were included if there was enough information in the C-unit to determine whether a word was used in an appropriate semantic context. Scores were calculated as the proportion of C-units containing each word category. Approximately 20% of the narrative transcripts from each book were coded independently by a second coder to assess reliability. Point-by-point inter-coder agreement averaged 96% (range = 83–100%) for *Frog Goes to Dinner* and 97% (range = 86–100%) for *Frog on His Own*.

TABLE 3 | Average point-by-point inter-coder agreement for story grammar elements.

	Frog Goes to Dinner (%)	*Frog on His Own* (%)	Total (%)
Initiating events	97.78	95.00	96.47
Attempts	91.11	100.00	95.29
Outcomes	91.11	92.50	91.76
Reactions	91.11	97.50	94.12

Mean Length of Utterance (MLU)

We used SALT software to compute participants' MLU (i.e., mean length of C-units) in morphemes from each participant's narrative transcript. Only complete and fully intelligible C-units were included in this computation. Thus, abandoned C-units and C-units with unintelligible segments were excluded because it is not possible to determine how many morphemes were produced within an unintelligible segment.

Leiter International Performance Scale-Revised (Leiter-R; Roid and Miller, 1997)

The Leiter-R is a standardized measure of nonverbal cognition that is nonverbal in administration and in participant response method. We used growth scores from the Brief IQ screener as a metric of nonverbal cognitive ability level for participant matching. Growth scores are scaled corrections of raw scores that take into account item difficulty but reflect absolute ability level rather than an age-based norm. This is of particular relevance for individuals with intellectual disability who may perform at the floor level of standard scores (Hessl et al., 2009). For ease of interpretation, however, we also report standard scores in the participant descriptives. The Leiter-R Brief IQ screener correlates with the Wechsler Intelligence Scale for Children, Third Edition (Wechsler, 1991) at $r = 0.85$, and reported reliability of the Leiter-R is $r = 0.88$. The Leiter-R is normed for ages 2–21 years.

Test for Reception of Grammar, Second Edition (TROG-2; Bishop, 2003)

The TROG-2 is a standardized measure of receptive syntax. Participants were instructed to point to pictures that best represented phrases or sentences spoken by an examiner. Due to extensive floor effects on standard scores, we report raw scores in the description of the sample characteristics of our participant groups. Reported internal consistency reliability of the TROG-2 is 0.88.

RESULTS

Sample Descriptive Characteristics

See **Table 1** for descriptive characteristics of our sample by participant group. There was no significant difference between the participants with DS and those with FXS in terms of chronological age ($t_{(43)} = 0.940$, $p = 0.353$) or nonverbal IQ ($t_{(43)} = -0.866$, $p = 0.391$). There also was no significant difference in MLU between the participants with DS and those with FXS, $t_{(43)} = -0.080$, $p = 0.937$. The participants with DS, however, displayed significantly lower MLUs than the TD participants, $t_{(44)} = -2.236$, $p = 0.030$. In terms of receptive syntax (i.e., TROG-2 raw scores), there was not a statistically significant difference between the groups with DS and FXS, $t_{(43)} = -0.437$, $p = 0.664$. The participants with DS, again however, exhibited significantly lower receptive syntax abilities than the TD participants, $t_{(44)} = -4.132$, $p < 0.001$.

Macrostructural Analyses

Story Grammar Organization

We used a nested regression model to examine the relation of diagnostic group to overall story grammar organization scores and to evaluate the contribution of MLU. Using DS as the reference group, we used dummy codes so that the binary variable "TD" represented the TD-DS comparison and the binary variable "FXS" represented the FXS-DS comparison. These diagnostic group variables were included in Step 1 of the regression model, and MLU was included in Step 2. An examination of residuals indicated no major violations of the assumptions of linear regression. The resulting model was significant, $F_{(3,67)} = 34.552$, $p < 0.001$, $R^2 = 0.618$, with the full model accounting for 62% of the variance in story grammar scores.

In the first step, diagnostic group accounted for a significant amount of the variance in the model, $F_{(2,67)} = 3.219$, $p = 0.046$, $R^2 = 0.090$. An examination of the standardized coefficients indicated that the TD-DS contrast was significant ($\beta = 0.331$, $p = 0.018$), but the FXS-DS contrast was not significant ($\beta = 0.077$, $p = 0.572$); thus, the TD group had significantly higher story grammar scores than the group with DS, but there was no significant difference in scores between the groups with FXS and DS.

The addition of MLU in the second step also accounted for significant unique variance to the model, $F\text{-}change_{(1,64)} = 88.547$, $p < 0.001$, R^2 change = 0.528. An examination of the standardized coefficients revealed that with MLU added into the model ($\beta = 0.765$, $p < 0.001$), the diagnostic group contrasts were no longer significant (TD-DS $\beta = 0.087$, $p = 0.350$; FXS-DS $\beta = 0.068$, $p = 0.445$). Thus, after accounting for MLU, the difference in story grammar scores between the TD group and the group with DS was no longer significant. See **Table 4** for story grammar scores by participant group.

Inclusion of Story Grammar Elements

To further analyze story grammar organization in the participants' narratives, we explored whether there was a difference among the participant groups in their inclusion of the

TABLE 4 | Primary analyses: narrative scores by participant group.

	DS *Mean (SD)* *Range*	FXS *Mean (SD)* *Range*	TD *Mean (SD)* *Range*
Macrostructural variables[a]			
Story grammar organization	6.52 (5.70)	7.36 (3.91)	10.09 (5.10)
	0–19	1–14	0–17
Microstructural variables[b]			
Verb use	0.50 (0.28)	0.61 (0.22)	0.74 (0.18)
	0.00–1.00	0.24–0.96	0.39–0.98
Adverb use	0.19 (0.18)	0.19 (0.16)	0.35 (0.23)
	0.00–0.76	0.00–0.51	0.04–0.78
Conjunction use	0.03 (0.06)	0.03 (0.05)	0.07 (0.10)
	0.00–0.18	0.00–0.15	0.00–0.41

[a]*Number of episodic elements expressed (maximum = 31).* [b]*Proportion of C-units including the word category.*

different types of story grammar elements. For these analyses, we examined the number of episodes (0–5) in which participants included each type of story grammar element; however, because internal responses and plans were rarely mentioned by any of our participants, we did not analyze them further. For the remaining elements, we used nonparametric analyses because of the limited range of scores and the violation of the assumption of normality in their distributions. See **Table 5** for median scores by element type for each group.

Settings

We used a 2 × 3 chi-square analysis to determine if there was a difference among participant groups in whether or not they mentioned a setting in their narratives. This analysis was not statistically significant, $X^2_{(2)} = 1.434$, $p = 0.488$, indicating no differences among groups in their use of a setting.

Initiating Events

A Kruskal-Wallis test, used to explore whether there was a difference among participant groups in how many Initiating Events they mentioned, was not statistically significant, $H_{(2)} = 4.149$, $p = 0.126$, indicating no between group differences.

Attempts

A Kruskal-Wallis test, used to explore whether there was a difference among participant groups in how many Attempts they mentioned, was marginally significant, $H_{(2)} = 5.525$, $p = 0.063$. Given the exploratory nature of these analyses, we conducted one-tailed Mann-Whitney *post hoc* analyses to explore whether there were fewer Attempts used by the group with DS compared to the TD group or the group with FXS. Results indicated that the group with DS used significantly fewer Attempts than the TD group ($U = 179.000$, $Z = -1.922$, $p = 0.028$) but not the group with FXS ($U = 232.500$, $Z = -0.489$, $p = 0.315$).

Outcomes

A Kruskal-Wallis test, used to explore whether there was a difference among groups in how many Outcomes they mentioned, was not statistically significant, $H_{(2)} = 3.129$, $p = 0.209$, indicating no group differences.

TABLE 5 | Exploratory analyses: use of story grammar element type by participant group.

	DS *Median (Range)*	FXS *Median (Range)*	TD *Median (Range)*
Setting[a]	34.8% used	52.2% used	45.5% used
Initiating Event[b]	3 (0–5)	3 (0–5)	4 (0–5)
Internal Response[b]	0 (0–1)	0 (0–1)	0 (0–2)
Plan[b]	0 (0–1)	0 (0–0)	0 (0–1)
Attempt[b]	1 (0–4)	1 (0–3)	2 (0–4)
Outcome[b]	1 (0–4)	1 (0–3)	1 (0–4)
Reaction[b]	1 (0–5)	2 (0–4)	2 (0–5)

[a]Scored as present/absent for entire story. [b]Scored as present/absent for each of the five episodes.

Reactions

A Kruskal-Wallis test, used to explore whether there was a difference among groups in the number of Reactions mentioned, was not statistically significant, $H_{(2)} = 4.318$, $p = 0.115$, again indicating no group differences.

Microstructural Analyses

Use of Grammatical Word Categories

To determine whether there were group differences in the use of the different grammatical word categories (proportionate to the total number of C-units produced), we created three nested regression models: one predicting verb use, one predicting adverb use, and one predicting conjunction use. For each model, with DS as the reference group, the dummy-coded binary variables of diagnostic group ("TD" and "FXS") were the independent variables. Again, we included MLU in the second step of the model to evaluate its contribution. An examination of residuals indicated no major violations of the assumptions of linear regression. See **Table 4** for proportions of word category use by participant group.

Verb Use

The model predicting verb use was significant, $F_{(3,67)} = 45.601$, $p < 0.001$, $R^2 = 0.681$, accounting for 68% of the total variance. In the first step, group contributed significant variance, $F_{(2,67)} = 6.008$, $p = 0.004$, $R^2 = 0.158$. The standardized coefficients revealed that the TD-DS contrast was significant ($\beta = 0.457$, $p = 0.001$), but the FXS-DS contrast was not ($\beta = 0.216$, $p = 0.104$), indicating a significantly lower rate of verb use by the group with DS relative only to the TD group. The inclusion of MLU in the second step accounted for a significant amount of additional variance in the model, $F\text{-}change_{(1,64)} = 105.122$, $p < 0.001$, R^2 change = 0.524. With MLU in the model ($\beta = 0.762$, $p < 0.001$), both diagnostic group contrasts became significant (TD-DS $\beta = 0.214$, $p = 0.014$; FXS-DS $\beta = 0.207$, $p = 0.013$). Thus, after accounting for MLU, the rate of verb use was significantly lower in the group with DS relative to both the TD group and the group with FXS.

Adverb Use

The model predicting adverb use was significant, $F_{(3,67)} = 25.619$, $p < 0.001$, $R^2 = 0.546$, accounting for 55% of the variance. In the first step, group accounted for a significant portion of the variance, $F_{(2,67)} = 5.159$, $p = 0.008$, $R^2 = 0.137$. The standardized coefficients revealed that the TD-DS contrast was significant ($\beta = 0.359$, $p = 0.009$), but the FXS-DS contrast was not ($\beta = -0.021$, $p = 0.873$), indicating a significantly lower rate of adverb use by the group with DS relative only to the TD group. The inclusion of MLU in the second step accounted for a significant amount of additional variance in the model, $F\text{-}change_{(1,64)} = 57.560$, $p < 0.001$, R^2 change = 0.409. The standardized coefficients revealed that, with MLU in the model ($\beta = 0.673$, $p < 0.001$), neither diagnostic group contrast remained significant (TD-DS $\beta = 0.145$, $p = 0.156$; FXS-DS $\beta = -0.029$, $p = 0.765$).

Conjunction Use

The model predicting conjunction use was significant, $F_{(3,67)} = 15.385$, $p < 0.001$, $R^2 = 0.419$, accounting for 42% of the total variance. In the first step, however, group did not contribute significant variance, $F_{(2,67)} = 1.537$, $p = 0.223$, $R^2 = 0.045$, indicating that neither the TD-DS ($\beta = 0.203$, $p = 0.151$) nor the FXS-DS ($\beta = -0.018$, $p = 0.895$) group differences in conjunction use was significant. The inclusion of MLU in the second step accounted for a significant amount of variance in the model, $F\text{-}change_{(1,64)} = 41.179$, $p < 0.001$, R^2 change = 0.374. With MLU in the model ($\beta = 0.644$, $p < 0.001$), the group contrast variables were still not significant (TD-DS $\beta = -0.002$, $p = 0.985$; FXS-DS $\beta = -0.236$, $p = 0.814$). However, it should be noted that across participant groups, conjunction use was very low (**Table 4**).

DISCUSSION

The present study was designed to examine the macrostructrual and microstructural aspects of narratives produced by children and adolescents with DS. Narrative is a foundational skill for learning to use language to interact with others (McCabe and Bliss, 2003; Reed and Spicer, 2003) and is a scaffold for the acquisition of literacy-related skills and academic achievement (Dickinson and McCabe, 2001). Thus, our study was designed to inform work on the behavioral phenotype of DS and provide insights into potential targets for interventions that could have positive consequences for the daily functioning of individuals with DS.

Unlike previous studies in this area, we focused on the mastery of the internal organization of the episodes that serve as the building blocks of a story. In general, individuals with DS expressed fewer of the elements of episodic structure than did younger TD children of similar nonverbal cognitive levels, suggesting that this aspect of narrative macrostructure is especially impaired in DS. We also found, however, that the difference in expression of episodic elements between youth with DS and TD children was eliminated once the difference in MLU, or syntactic competence, was controlled. This finding suggests that individuals with DS have acquired the conceptual knowledge needed to express the key story elements (at least to the level expected for their nonverbal cognitive ability), but that their limited expressive syntactic abilities limit their ability to put that knowledge into words during the course of telling a story. This conclusion is consistent with the findings of previous studies suggesting that individuals with DS can sometimes express conceptually more mature narratives than TD peers when expressive abilities are equated through participant selection and or statistical control (Boudreau and Chapman, 2000; Miles and Chapman, 2002). In the current study, however, this result occurred despite the fact that, in our coding scheme, a participant was awarded credit for a story element even if it was communicated over two or three short utterances rather than in one utterance. It would appear, then, that there is a need for interventions targeting narrative language competence in DS and that such interventions should provide models of, and practice with, a range of linguistic options for expressing episodic structure.

In typical development, children begin to use individual story grammar elements in their narratives during the early preschool years. Most 3-year-olds only describe isolated pictures, mentioning only the most salient aspects of the story in a fragmented manner. Older preschool-aged children begin to communicate event sequences and connect the initiating event with an outcome in the story, sometimes also mentioning character actions that mediate the initiating event and the outcome/consequence. By around 5 or 6 years of age, children can formulate temporally organized event sequences and are able to communicate overarching story themes. Story grammar organization continues to progress during the school-age years as children develop a stronger cognitive framework for event sequencing and for talking about character goals and plans (Karmiloff-Smith, 1981; Bamberg, 1987; Bamberg and Marchman, 1990; Reilly, 1992; Berman and Slobin, 1994). The findings from our study suggest that in DS, story grammar organization may develop closely with and rely critically on expressive grammar.

Our exploratory analyses revealed that the difficulty observed in story grammar in the participants with DS may be centered on the expression of Attempts, which are in some ways the core of an episode, representing the actions taken by story characters to deal with the problem or dilemma that launched the episode. Because Attempts are actions that are motivated by a character's goals and other internal states, the results suggest that individuals with DS have difficulty talking about others' perspectives and intentions, an idea compatible with the growing body of literature on the social behavioral phenotype of DS (Fidler et al., 2005; Fidler, 2006; Cebula et al., 2010; Hahn et al., 2013). The lack of communication of character actions could also stem from a difficulty in verb production that has been observed in DS in other language sampling contexts (Hesketh and Chapman, 1998; Michael et al., 2012), as verbs are necessary to communicate character actions. Although verbs are also important for communicating other story grammar elements (e.g., plans or reactions), action verbs in particular are needed to encode and express character attempts/actions. Regardless of explanation, it would appear that interventions targeting narrative language competence in DS should include an emphasis on the expression of Attempts. This could include focusing on skills such as event sequencing or perspective taking, and narrative storytelling provides an optimal context for scaffolding such skills.

We also found that the narratives of individuals with DS did not differ from those of individuals with FXS, at least in terms of the aspects of narrative macrostructure we examined. This finding suggests that the impairments in narrative macrostructure we examined are not specific to DS, but may be associated with intellectual disability more generally. Beyond their intellectual disability, individuals with DS and FXS share a delay in spoken language. Although there are marked differences in the specific language profiles observed across the two disorders (e.g., Abbeduto et al., 2003), the findings

from this study suggest a shared deficit in the use of story grammar.

In terms of narrative microstructure, we examined the expression of three major syntactic categories of words—verbs, adverbs, and conjunctions—all of which are critical for the expression of episodic structure as well as other dimensions of narrative macrostructure. More specifically, verbs allow children to talk about overt character actions (e.g., *run*, *catch*, *jump*) as well as character psychological states, including their plans and goals (e.g., *want*, *hope*) or emotional reactions (e.g., *laugh*, *cry*). Conjunctions aid event sequencing by linking events in temporal (e.g., "He took a drink *after* he jumped in the glass") and causal (e.g., "He fell over *because* the bee stung his tongue") relationships. Adverbs (i.e., words that describe where, when, how, etc.), much like conjunctions, also play an important role in accurately describing events in the context of place and time (e.g., *over there*, *next*) as well as allowing the child to use evaluative devices that enable the speaker to make comments about the story to the listener (e.g., "He *really* didn't like it", "The frog *always* got in trouble").

Individuals with DS were less likely to use adverbs and verbs in their stories than were their TD cognitively matched peers. After controlling for variation in MLU, the group difference in the rate of adverb use was no longer significant. Controlling for MLU, however, did not eliminate the DS-TD difference in rate of verb use. Further, when controlling for MLU, the difference between the groups with DS and FXS also became significant, with the participants with DS showing a lower rate of verb use. This suggests that beyond their general impairment in expressive grammar, individuals with DS exhibit a specific deficit in verb production that may contribute to their unique behavioral phenotype rather than being general to intellectual disability. Indeed, problems in verb mastery have been documented in DS in other studies as well (Hesketh and Chapman, 1998; Chapman, 2003), and our findings extend this to the context of narrative storytelling from a book and by documenting an impairment not shared by those with FXS. Given the action-oriented nature of Attempts, it is likely that limitations in expressive mastery of verbs may be contributing to the macrostructure impairments displayed by individuals with DS. Thus, interventions designed to improve narrative competence in this population should also pay particular attention to modeling new action verbs in the service of expressing narrative content. This would provide individuals with DS with the linguistic tools needed to express the key story elements, particularly Attempts, in their narratives, thus improving their narrative language at the macrostructural level as well.

The finding regarding the relative role of MLU to group differences in verb use further suggests that for individuals with DS, other mechanisms may be driving their development of verb use. For example, it could be that the specific weakness in phonological memory that is characteristic of many individuals with DS plays a role in their ability to learn verbs, which often appear in the middle of a spoken sentence and thus are more difficult to encode (see Naigles et al., 1995; but see Miolo et al., 2005). Another possibility is that a difficulty in abstract learning may be driving this deficit. That is, the abstract nature of verbs requires children to learn the word they hear by mapping it to a transitory action they observe, a much less concrete task than mapping a label (i.e., a noun) to an object that remains in front of them. Before any conclusions can be drawn, however, more research is needed to identify such potential predictors of verb learning in DS. Because verbs are so integral to the ability to effectively communicate events and personal experiences to others, this is an area worthy of further investigation.

Beyond contributing to a better understanding of narrative abilities in DS, our study also highlights a new approach to measuring both macrostructural and microstructural narrative abilities of individuals with intellectual disability that can be used in phenotypic research as well as for measuring change over time (e.g., in response to a language intervention). With the recent focus on developing outcome measures that are appropriate for individuals with intellectual disability across a wide age range (e.g., Berry-Kravis et al., 2013b), the Narrative Task (Abbeduto et al., 1995) employed in this study has received much attention. This task provides a naturalistic context for measuring spoken language (i.e., storytelling from a picture book), while also providing a standardized context for administration. Because it is not subject to the same floor effects and compliance problems that occur with many standardized assessments of spoken language in these populations, it is an ideal candidate for use as an outcome measure. In fact, several research studies have documented its utility as such, showing that the expressive language measures derived from this task (e.g., MLU, vocabulary diversity, talkativeness, etc.) can discriminate typical from atypical populations, distinguish different genetic syndromes associated with intellectual disability, and show excellent test-retest reliability (Abbeduto et al., 1995; Finestack and Abbeduto, 2010; Kover et al., 2012; Berry-Kravis et al., 2013a). The current study goes beyond the standard expressive language measures that can be derived from the Narrative Task and demonstrates its ability to detect individual differences, as well as differences among typical and atypical samples, in aspects of narrative language competence at the macrostructural and microstructural levels of analysis.

There are, however, limitations to the present study. First, the small sample sizes of the participant groups and the exploratory nature of the analyses by story grammar element type suggest that the results should be interpreted with caution and are in need of replication. Future research should also test the generalizability of the findings to the broader population with DS (e.g., other age ranges and ability levels). Although the age range of participants in the present study is ideal for examining narrative language competence, in particular, the results may be less applicable to individuals with DS who are younger and/or less verbal than the present sample. Furthermore, we did not screen for comorbid diagnoses such as autism spectrum disorder that could also affect the generalizability of the results. Finally, participant groups were not matched on sex, another important factor to consider in future work.

In sum, this study extended prior work on narrative language in DS by taking a new approach to measuring their macrostructural and microstructural narrative abilities and by adding a same-age comparison group of individuals with intellectual disability of another origin (i.e., FXS). Importantly, this study provides a new method for researchers to capture individual differences across a wide range of ages and ability levels of individuals with intellectual disability, including those with DS. By no means, however, did this study capture all of the macrostructural and microstructural narrative language abilities of individuals with DS. For example, the use of evaluative devices that engage the listener, such as sound effects or character dialog, would provide additional insight into their story-telling abilities. Furthermore, researchers should consider the use of inferential language (e.g., mental state language; predictions; causal referencing) in the narratives produced by individuals with DS, as this would provide more information regarding their perspective taking and abstract reasoning skills. Finally, researchers should also consider using videos to capture non-linguistic communication acts (e.g., gestures or facial expressions) that children with DS may be using to communicate their stories to a listener. Ultimately, data on narrative provide an exciting new avenue for intervention, both in terms of informing clinicians where to target during intervention to promote spoken language development and in equipping them with a way to capture change in the use of those skills over time within the naturalistic context of shared book reading.

AUTHOR CONTRIBUTIONS

MMC and ASM conceptualized the study's design, developed the coding schemes reported in the manuscript, and had primary roles in data analysis and interpretation and in drafting the manuscript. LMB helped develop the coding scheme, assisted with data analysis, and helped draft the manuscript. LA participated in the study's conception and design, assisted in data interpretation and drafting the manuscript, and provided mentorship throughout the project. All authors have read and approved the final version of this manuscript.

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Semantic Verbal Fluency Pattern, Dementia Rating Scores and Adaptive Behavior Correlate With Plasma $A\beta_{42}$ Concentrations in Down Syndrome Young Adults

*Laura Del Hoyo[1,2], Laura Xicota[1,3,4], Gonzalo Sánchez-Benavides[1], Aida Cuenca-Royo[1], Susana de Sola[1,3], Klaus Langohr[1,5], Ana B. Fagundo[6,7], Magí Farré[1,2], Mara Dierssen[3,4,8] and Rafael de la Torre[1,4,7]**

[1] Neurosciences Research Program, Integrative Pharmacology and Systems Neuroscience Research Group, IMIM-Institut de Hospital del Mar d'Investigacions Mèdiques, Barcelona, Spain, [2] Departamento de farmacología, Universitat Autònoma de Barcelona, Barcelona, Spain, [3] Systems Biology Program, Cellular and Systems Neurobiology, Centre for Genomic Regulation (CRG), The Barcelona Institute of Science and Technology, Barcelona, Spain, [4] Department of Experimental and Health Sciences, Universitat Pompeu Fabra, Barcelona, Spain, [5] Department of Statistics and Operations Research, Universitat Politècnica de Barcelona/BarcelonaTech, Barcelona, Spain, [6] Department of Psychiatry, University Hospital of Bellvitge-IDIBELL, Barcelona, Spain, [7] CIBER Fisiopatología Obesidad y Nutrición (CIBERObn), Instituto Salud Carlos III, Madrid, Spain, [8] CIBER de Enfermedades Raras (CIBERER), Instituto Salud Carlos III, Madrid, Spain

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****Correspondence:***
Rafael de la Torre
rtorre@imim.es

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Down syndrome (DS) is an intellectual disability (ID) disorder in which language and specifically, verbal fluency are strongly impaired domains; nearly all adults show neuropathology of Alzheimer's disease (AD), including amyloid deposition by their fifth decade of life. In the general population, verbal fluency deficits are considered a strong AD predictor being the semantic verbal fluency task (SVFT) a useful tool for enhancing early diagnostic. However, there is a lack of information about the association between the semantic verbal fluency pattern (SVFP) and the biological amyloidosis markers in DS. In the current study, we used the SVFT in young adults with DS to characterize their SVFP, assessing total generated words, clustering, and switching. We then explored its association with early indicators of dementia, adaptive behavior and amyloidosis biomarkers, using the Dementia Questionnaire for Persons with Intellectual Disability (DMR), the Adaptive Behavior Assessment System-Second Edition (ABAS-II), and plasma levels of Aβ peptides ($A\beta_{40}$ and $A\beta_{42}$), as a potent biomarker of AD. In DS, worse performance in SVFT and poorer communication skills were associated with higher plasma $A\beta_{42}$ concentrations, a higher DMR score and impaired communication skills (ABAS–II). The total word production and switching ability in SVFT were good indicators of plasma $A\beta_{42}$ concentration. In conclusion, we propose the SVFT as a good screening test for early detection of dementia and amyloidosis in young adults with DS.

Keywords: Down syndrome, Alzheimer's disease, semantic verbal fluency, switching, Aβ, amyloid precursor protein, communication skills, DMR

INTRODUCTION

Adults with Down syndrome (DS) have a high risk for the development of early onset dementia and invariably develop senile plaques, composed of β-amyloid peptide (Aβ), indistinguishable from the histopathology of sporadic Alzheimer's disease (AD; Rumble et al., 1989). Plaques can be found in almost all adults from 35–40 years of age (Zigman et al., 2008), and the presence of Aβ oligomers can be detected as early as during fetal development (Teller et al., 1996; Lott and Dierssen, 2010), although the clinical symptoms clearly differ from those observed in AD in general population.

The increase in lifespan in the DS population has made the early detection of dementia of Alzheimer's type a major objective of researchers and clinicians. In the general population impairments in semantic fluency exist prior to the clinical diagnosis of AD (Vogel et al., 2005). Specifically, patients with AD exhibit important deficits in both semantic and phonemic fluency, being the former the most impaired (Cerhan et al., 2002; Canning et al., 2004; Henry et al., 2004; Taylor et al., 2005). Thus, alterations in clustering and switching abilities during the performance of a semantic verbal fluency task (SVFT) are considered early predictors of the development of AD in the general population (Palmer et al., 2003; Fagundo et al., 2008).

Conversely, even though research on DS has substantiated language and verbal fluency as one of the most impaired domains (Palmer et al., 2003) influencing cognitive-related outcomes and daily living functionality (Edgin et al., 2010; de Sola et al., 2015), there is a paucity of information about the verbal fluency pattern in DS young adults. To our knowledge, only two studies have reported the semantic verbal fluency pattern (SVFP) in DS, one in pediatric population, in which a reduced productivity of words and switching was shown in DS subjects compared to age-matched controls (Nash and Snowling, 2008) suggesting less efficient retrieval strategies. The second one in adult population with learning disabilities (Rowe et al., 2006), showed reduced word production, but the responses were only analyzed accounting for total number of correct words, regardless of performance in retrieval strategies such as clustering and switching.

To date AD conversion in aged DS subjects is mainly analyzed by measuring plasma Aβ concentrations. Several studies have shown increased concentrations of both $A\beta_{40}$ and $A\beta_{42}$ in young DS compared to control population (Mehta et al., 2003; Head et al., 2011) and most found higher concentrations of $A\beta_{42}$ in those DS individuals that were either demented or developed dementia at follow-up (Schupf et al., 2007; Prasher et al., 2010; Coppus et al., 2012). Some correlations have also been found between high $A\beta_{40}$ plasma levels and dementia status, and between increases in $A\beta_{40}$ and decreases of $A\beta_{42}$ and risk of dementia (Schupf et al., 2007, 2010; Head et al., 2011; Coppus et al., 2012). Interestingly, most studies report no correlation between age and $A\beta_{42}$ levels (Prasher et al., 2010; Head et al., 2011).

In the current study, we aimed at characterizing the SVFP including clustering and switching abilities in adults with DS in comparison to age-matched general population. To this aim, we used the SVFT that requires verbal abilities, search and retrieval skills, adequate processing speed, and the capacity to inhibit inappropriate responses (Henry and Phillips, 2006). The total number of words and the clustering, which measures the way these words are grouped by different semantic categories (i.e., pets, farm, aquatic animal etc.), provide an indirect measure of the organization of semantic representations. On the other hand, the use of retrieval strategies, such as switching from one semantic category group of words to a new one, yields information about the set shifting ability, an executive skill related to the integrity of the frontal lobes. We then explored the association of SVFT performance with early indicators of dementia, adaptive behavior and amyloidosis biomarkers (Aβ40 and Aβ42).

MATERIALS AND METHODS

Participants

The sample was drawn from the baseline visit of a clinical trial (TESDAD Study ClinicalTrials.gov Identifier: NCT01699711). Participants enrolled in our cross-sectional study (n = 50) were young adults (aged 17–34 years) of both genders with DS (complete trisomy 21, mosaic or translocation). Subjects with neurological disease other than DS (epilepsy, cerebral palsy, hemiplegia, central nervous system infection with neurological deficit), relevant medical disease, unstable co-morbid mental disorder (anxiety disorder, depression, obsessive compulsive disorder), or undergoing any treatment that could interfere with cognitive function or alter key biomarker analyzed were excluded from the study. Also, exclusion criteria included subjects with severe language deficit (significant speech and/or comprehension limitations), behavioral disturbances and/or poor level of collaboration during the assessment but no subjects were excluded from the analysis by this criterion.

To determine the gap in cognitive performance between DS subjects and healthy adults a comparison group, matched for age (mean age: 22.6 ± 3.8) was included 59 young healthy adults of both genders. These participants were assessed in previous neuropsychological studies (de Sola et al., 2008; Fagundo et al., 2010). Healthy volunteers were excluded if they had neurological or relevant medical diseases, or if they had been diagnosed with a psychiatric disorder following DSM-IV criteria. Whilst prevailing methodology compares DS subjects to healthy controls of the same "mental age" to provide an index of global level of mental functioning (Edgin et al., 2010; Finestack and Abbeduto, 2010), this perspective is not useful for characterizing specific capacities (Costanzo et al., 2013; de Sola et al., 2015).

The study was conducted in accordance with the Declaration of Helsinki and Spanish laws concerning data privacy. The protocol was approved by the Ethical Committee of the Parc de Salut Mar of Barcelona (CEIC-PSMAR). Upon arrival at the research center (Hospital del Mar Medical Research Institute-IMIM), participants, parents and legal guardians (in case of legal incapacitation) were informed of the ensuing protocol and they gave their written informed consent before participating.

Procedure

Semantic Verbal Fluency Pattern

We used the SVFT (Benton et al., 1976) as a measure of semantic memory and executive functioning. Three outcome variables were obtained: (i) the total number of correctly generated words in 60 s, and the percentage of words generated every 15 s; (ii) errors committed including intrusions (words not belonging to the specified semantic category), perseverations and repetitions (same words or same words with different endings); and (iii) clustering and switching measures that were obtained to determine the strategies used to perform the task. Mean cluster size was the main dependent variable for clustering, whereas number of switches was the main dependent variable for switching (Troyer et al., 1997; Troyer, 2000). A cluster was defined as any series of two or more successively produced words belonging to the same semantic subcategory, determined *a priori* (Fagundo et al., 2010). Cluster size was computed by adding up series of words from the same subcategory starting from the second word within each cluster (i.e., a three-word cluster has a size of two). The number of switches was defined and computed as the number of times the participant changed from one cluster to another. Two clusters may also be overlapping, for example, from "farm animals" to "birds" in "cow–pig–chicken–pigeon–eagle." Here, one switch is made between the cluster "cow–pig–chicken" and "chicken–pigeon–eagle." The computation of number of switches included single-word clusters. An inter-rater reliability analysis was performed and the reliability studied by means of the intra-class correlation coefficient (ICC) was high with values ranging from 0.89–0.98.

Intellectual Quotient IQ

The intellectual quotient estimation was assessed with The Kaufman Brief Intelligence Test (Kaufman and Kaufman, 1990).

Functional Measures

The Adaptive Behavior Assessment System-Second Edition (ABAS-II, adult version; Harrison and Oakland, 2003) for evaluating adaptive skills in people with intellectual disabilities and the Dementia Questionnaire for Persons with Intellectual Disability (Evenhuis et al., 2006) previously named Dementia Questionnaire for persons with Mental retardation. The DMR is a self-reported questionnaire about daily living abilities, which measures specific memory and orientation cognitive skills and social deterioration as a result of dementia and/or severe sensory or psychiatric problems. It consists of 50 items and eight subscales. Combined scores on the first three subscales (Short-term memory, Long-term memory and Orientation) are presented as the Sum of Cognitive Scores (SCS). Combined scores on subscales four through to eight (Speech, Practical skills, Mood, Activity and Interest, and Behavioral disturbance) are presented as the Sum of Social Scores (SOS). Higher scores in DMR reflect a worse state, while higher punctuations in ABAS-II reflect a better adaptive behavior.

Both questionnaires were given to the caregivers for completion while participants completed the neuropsychological testing. We ensured they understood how to complete the questionnaires and solved all doubts before and after completion, and checked that all questions were filled.

Plasma Aβ Measurement

Overnight fasting blood samples were collected on site by a qualified nurse, during the morning hours. The blood was drawn into 8 mL Heparin Lithium tubes (B&D, UK), centrifuged at 4°C for 15 min at 3000 rpm, and the plasma was distributed in aliquots and stored at −70°C until analysis. Samples (only for DS subjects) were analyzed for plasma Aβ concentrations, using Inno-bia Plasma Aβ forms (Aβ40 and Aβ42, truncated Aβ40 and Aβ42 not reported) assay (Innogenetics, Fujirebio) following the manufacturer instructions. The plaques were read in a Bio-Plex 200 Systems (Bio-Rad) instrument, and the standard curves were fitted using the provided software (Bioplex Manager 6.1).

Statistical Analysis

Results are described by means of measures of both central tendency (mean and median) and variability (standard deviation and range) for numeric variables, and absolute and relative frequencies for categorical variables. In the case of the IQ, only the median is reported because no distinction is made of values below 40. The differences between DS and healthy groups with respect to semantic verbal fluency performance are quantified by means of the standardized mean difference (Cohen's d). The computation of all correlations of interest was done using Pearson's correlation coefficient. ANCOVA models were used to study the associations in DS between semantic verbal fluency outcomes, other cognitive and functional measures, and AD biomarkers, on one hand, and gender, IQ, and age, on the other hand. For these analyses, the IQ was categorized into two groups: mild/moderate ($IQ \geq 40$) and severe ($IQ < 40$) within the range of ID level.

Statistical significance was set at 0.05. All statistical analyses were performed using the statistical software packages SPSS (Version 18.0; SPSS Inc., Chicago, IL, USA) and R (Version 3.2.1; The R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Descriptive Demographic and Clinical Data of the Participants

In our DS sample, 48% individuals were male and the mean age was 23.6 years (standard deviation (SD): 4.5 years; range: 17–34 years). The median IQ was 41 (38% with IQ less than 40) and a maximum IQ of 70, whereas the mean K-BIT standardized score was 103 (SD: 14.9; range: 80–151). In terms of gender, the median IQ among males was 40 (IQ less than 40: 37.5%; maximum: 66) and among females 41.5 (IQ less than 40: 38.5%; maximum: 70), whereas the mean K-BIT standardized scores were 101 (SD: 15.6; range: 80–144) and 105 (SD: 14.3; range: 80–151), respectively. Concerning the DS karyotypes, the sample showed the usual proportion for this population, with most individuals with full trisomy 21 (48 simple trisomies, one translocation, and one mosaic). Regarding Aβ plasma concentrations, the mean $A\beta_{40}$

concentration was 270.9 pg/mL [SD: 50.8; range: 174–439.3] and the mean $A\beta_{42}$ was 41 pg/mL [SD: 10; range: 21.5–60.9].

Semantic Verbal Fluency Performance in DS Individuals Compared to Standard Norms

Descriptive analyses, Cohen effect size differences (*d*), and confidence intervals (95% CI) of fluency task performance in DS individuals and age-matched standard norms are summarized in **Table 1**. Our results show that in DS switching correlated more strongly than clustering with the total number of words generated (See **Table 2**). We found no correlation between the percentage of words produced in the first 15 and last 45 s, with the total number of words. The mean percentage of words produced in the first 15 s was 37.8%.

On the contrary, in age-matched healthy population there is a stronger correlation between clustering and the total number of generated words, while there is no correlation between switching and the total number of words produced. Besides, we found a negative correlation between the percentage of words produced in the first 15 s (mean *percentage* = 39.6) and the total number of words, and a positive correlation between the percentage of words produced during the last 45 s and the total number of words, indicating a more extensive lexicon in this population.

Association between IQ, Gender and Age, and Semantic Verbal Fluency Outcomes in DS

ANCOVA models were applied to analyze the association between the IQ, gender, and age and the semantic verbal fluency performance of DS individuals. As shown in **Table 3**, no statistically significant associations were found between IQ, age and gender, and the verbal fluency pattern in the DS group.

Association between IQ, Gender, and Age and AD Biomarkers in DS

ANCOVA models were applied to analyze the association between IQ, gender, and age, on one hand, and $A\beta_{40}$, $A\beta_{42}$, $A\beta_{42/40}$ plasma concentrations of DS individuals, on the other hand. We found a statistically significant association between IQ and $A\beta_{40}$. The negative parameter estimate indicates lower $A\beta_{40}$ concentrations among DS individuals of the same age and sex with an $IQ < 40$ compared with those with an $IQ \geq 40$ (**Table 4**).

Associations of $A\beta_{42}$ Concentration with Cognitive and Dementia Rating Functional Outcomes

ANCOVA models were applied to analyze the association between $A\beta_{42}$ concentration and both semantic verbal fluency outcomes and functional state among DS individuals. The models were adjusted for IQ, sex, and age (**Table 5**). Individuals with higher concentrations of $A\beta_{42}$ produced lower number of correct words and lower number of switches. Regarding adaptive behavior, subjects with higher $A\beta_{42}$ plasma concentrations had lower scores in the subscale "Communication skills" of the ABAS questionnaire. Concerning dementia rating, higher $A\beta_{42}$ plasma concentrations were associated with higher DMR total score; see **Figure 1** for graphical representations of the statistically significant associations.

Correlation between Fluency Measures and Functional Outcomes

The total number of words produced in 1 min and switching have both a positive correlation with communication skills and a negative correlation with the DMR total score. Furthermore, the total number of words produced in 1 min is positively correlated with the ABAS total score (**Table 6**).

DISCUSSION

Our study has found an association between the SVFP, dementia rates, and adaptive behavior related to communication skills in young adults with DS. Moreover, worse semantic fluency, higher dementia rates, and poor adaptive behavior and communication skills were associated to higher plasma concentrations of an AD biomarker ($A\beta_{42}$).

The observed associations between cognitive, functional, and biological parameters suggest that SVF assessment could be used a screening test for early detection of early symptoms of dementia DS. Furthermore, our study shows for the first time

TABLE 1 | Cognitive performance in DS individuals compared to standard norms.

	Down syndrome		Reference standard norms		Standardized mean differences	
Verbal fluency	**Mean *(SD)***	**Range *(min–max)***	**Mean *(SD)***	**Range *(min–max)***	***d***	***95%-CI***
Number of correct words in 60'	9.4 (4.1)	1–20	25.1 (5.7)	11–38	−3.13	[−3.69, −2.57]
Percentage of correct words 0–15'	39.2 (16.6)	0–100	39.6 (7.9)	25–54	−0.03	[−0.46, 0.4]
Percentage of correct words 16–30	28.1 (12.3)	0–50	22.7 (6.5)	14–39	0.53	[0.09, 0.96]
Percentage of correct words 31–45	16.4 (12.4)	0–50	18.6 (5.8)	5–32	−0.22	[−0.65, 0.21]
Percentage of correct words 46–60	17.1 (12.0)	0–60	18.6 (9.5)	0–46	−0.14	[−0.56, 0.29]
Number of switches	4.3 (2.5)	0–13	7.4 (2.1)	3–11	−1.4	[−1.82, −0.97]
Mean cluster size	1.1 (0.8)	0–3.3	2.8 (0.9)	1.4–6.6	−1.93	[−2.39, −1.46]

The standardized mean differences are calculated using Cohen's d. Age range: DS: 17–34, Reference standard norms 18–33 Sample size: DS: n = 51, Reference Standard norm: n = 59.

TABLE 2 | Correlation between fluency strategies and the total number of words produced (Pearson's correlation coefficient).

	Total correct words			
	Down syndrome		Reference standard norms	
	Correlation [95%-*CI*]	*p*-value	Correlation [95%-*CI*]	*p*-value
Number of switches	0.73 [0.57, 0.84]	<0.001	0.17 [−0.1, 0.41]	0.244
Mean cluster size	0.3 [0.02, 0.53]	0.039	0.49 [0.26, 0.67]	<0.001
Percentage of animals in the first 15 s	0.03 [−0.25, 0.31]	0.84	−0.54 [−0.73, −0.25]	0.001
Percentage of animals in the last 45 s	0.11 [−0.18, 0.38]	0.453	0.51 [0.22, 0.72]	0.001

clear differences in the SVFP of a DS young adult population compared to healthy age-matched individuals.

Fluency Deficits in Young Down Syndrome Adults

Impairment of verbal fluency, as estimated by lexical knowledge, is a feature of DS (Rowe et al., 2006). Our results showed a reduction of switching and cluster size as compared to the age-matched group, possibly due to a worse semantic knowledge. This profile is similar to the so called dysexecutive syndrome, described as a common pattern of dysfunction in executive functions such as planning, abstract thinking, flexibility, and behavioral control (Wilson et al., 1998). A dysexecutive syndrome has already been reported in DS (Rowe et al., 2006; Lanfranchi et al., 2010; de Sola et al., 2015), and has been related to the reduced volume of the prefrontal cortex reported in neuroimaging studies (Raz et al., 1995; White et al., 2003; Carducci et al., 2013), in particular affecting the anterior cingulate gyrus, medial, and dorsolateral prefrontal cortices (Contestabile et al., 2010; Lott and Dierssen, 2010). These areas actively contribute to mnemonic processing and executive control in euploid individuals (Braver et al., 2001; Wager and Smith, 2003; Blumenfeld et al., 2011), and, thus, the generalized impairment of high order frontal-dependent processes has a negative influence on SVFP which depends on both mnemonic and executive processes.

Similarly to what is observed in healthy population in our DS group the word production decreases significantly with time, although in the DS group we detected the wide individual variability typically shown in the DS population. The production decrease over time can be explained according to the model of lexical organization (Crowe, 1996), which states that there are two types of storages, namely: (1) a long-term storage ("topicon") which is readily accessible and contains common words and (2) a more extensive lexicon which is searched after the "topicon" is exhausted. Thus, successful performance on a verbal fluency task seems to be subjected to the effectiveness of both automatic and controlled processing (Crowe, 1998; Hurks et al., 2006). In our DS sample, subjects are not differentiating between using automatic processing and instead, they access to the pool of frequently used words, but when this is exhausted, they fail in using controlled attentional searching retrieval processes that involve executive strategies with high impact on total word production, such as switching. Word production in normative age matched population also decreases over time, paired with a high percentage of words produced in the first 15 s as reported in previous studies (Villodre et al., 2006). However, their topicon and lexicon are richer than DS due to better semantic knowledge (clustering) and retrieval strategies (switching).

Aβ Plasma Concentrations in Young DS Group

Regarding the plasma concentrations of amyloid peptides, few studies have measured the concentrations of such biomarkers in young adults (Mehta et al., 2003; Head et al., 2011), and those were performed in older populations. Compared to them, we obtained higher mean concentrations of $A\beta_{42}$ (41 ± 10 pg/mL), possibly due to the sensitivity of method we used. However, another study performed in younger DS subjects (mean 7.2 ± 3.8) obtained concentrations (31.6 ± 8.2 pg/mL) that were closer to our mean values (Mehta et al., 2007).

Contrary to previous studies in older DS populations (Prasher et al., 2010; Schupf et al., 2010; Head et al., 2011) that report a correlation of $A\beta_{42}$ with age, in ours this is not present suggesting that factors other than age are affecting the $A\beta_{42}$ production.

In light of our results, the impact of these biomarkers and their evolution pattern should be studied throughout adulthood in DS, and not only in the elderly.

TABLE 3 | Association between the verbal fluency pattern and the intellectual quotient (IQ), sex and age in DS individuals.

	IQ (<40 vs. ≥40)		Sex (Women vs. men)		Age	
Verbal fluency outcomes	**Estimate *(SE)***	***p***	**Estimate *(SE)***	***p***	**Estimate *(SE)***	***p***
Number of correct words in 60 s	−1.04 (1.18)	0.386	0.45 (1.17)	0.704	0.18 (0.13)	0.190
Number of switches	0.25 (0.73)	0.736	0.44 (0.72)	0.547	0.04 (0.08)	0.663
Mean cluster size	−0.36 (0.23)	0.112	−0.07 (0.22)	0.750	0.02 (0.03)	0.488

Parameter estimates, standard errors (SE), and p-values are obtained from ANCOVA models.

TABLE 4 | Association between Aβ concentrations and intellectual quotient (IQ), sex, and age in DS individuals.

Aβ concentrations	IQ (<40 vs. ≥40)		Sex (Women vs. men)		Age	
	Estimate *(SE)*	*p*	Estimate *(SE)*	*p*	Estimate *(SE)*	*p*
$A\beta_{42}$	−2.05 (2.97)	0.495	2.12 (2.94)	0.475	−0.21 (0.33)	0.527
$A\beta_{40}$	−38.2 (14.6)	0.012	19.9 (14.2)	0.167	−0.26 (1.59)	0.873
$A\beta_{40/42}$	0.007 (0.013)	0.564	0.004 (0.012)	0.758	−0.0002 (0.001)	0.893

Parameter estimates, standard errors (SE), and p-values are obtained from ANCOVA models.

TABLE 5 | Association between $A\beta_{42}$ concentration and cognitive and functional measure.

	Estimate *(SE)*	*p*
Cognitive performance in SVFT		
Number of correct words in 60'	−0.187 (0.052)	<0.001
Number of switches	−0.085 (0.035)	0.018
Mean cluster size	−0.006 (0.011)	0.582
Functional outcomes		
ABAS adaptive behavior: communication skills	−0.532 (0.158)	0.001
DMR total score	0.366 (0.113)	0.002

Parameter estimates, standard errors (SE), and p-values are obtained from ANCOVA models adjusted for IQ, age, and sex.

Association between Fluency Performance and Aβ Concentrations

A large subset of aged individuals with DS develop clinical features of AD and some studies have suggested deficits in executive function (Holland et al., 2000; Ball et al., 2006). In AD patients, AD was better predicted by the clustering ability in some reports (Fagundo et al., 2008), although others (Raoux et al., 2008) found a significant decline in switching along the early phase until the clinical diagnosis of AD dementia. In our DS population, switching and the total number of words are the verbal fluency markers that better correlate with the plasma $A\beta_{42}$ concentrations. This observation supports the hypothesis that impaired switching abilities could explain the early decline in semantic fluency performance in an early state of AD. Moreover, the association between AD biomarkers and verbal fluency pattern is supported by the correlation that we found between Aβ concentrations, dementia ratings (DMR), and communication skills. We observed that higher concentrations of $A\beta_{42}$ were associated to lower adaptive behavior and communication skills and higher DMR scores. In accordance, DMR can be considered useful detecting early symptoms of AD in DS. These results would also be in agreement with previous studies linking higher $A\beta_{42}$ plasma concentrations in elderly DS with dementia or the development of dementia (Schupf et al., 2007; Prasher et al., 2010; Coppus et al., 2012). Furthermore, DMR scores were inversely correlated with the SVFP. This is interesting because, in our study, positive correlations were found between communication skills, semantic verbal fluency, and switching, as discussed above. Communication abilities are a compilation of cognitive and social processes such as comprehension, expression, and empathy. Semantic verbal fluency seems to be part of this compilation of abilities involved in verbal expression as forming part of communication skills. In our case, the DS subjects are not demented, but there is a clear correlation between higher $A\beta_{42}$ and worse scores in functional variables that can be used to detect an early dementia state.

Limitations

The present study has several limitations. First, plasma measurements of Aβ concentrations remain controversial. Their high variability and lack of correlation with the observations of amyloidosis in the brain are some of the reasons leading some researchers to perform their measurements in CSF, that were not performed in our study. In our study, several peripheral tissues and cells, such as muscle and platelets, could be the source of peripheral Aβ (Toledo et al., 2014). However, in the context of clinical trials, as well as in clinical practice in general, it is worth improving the reliability of this blood measurement, as it is much less invasive than CSF extraction, as well as exploring its correlations with early cognitive symptoms of AD. Second, we only compared the SVFT between the DS group and the age-matched group. The rest of assessments as dementia rates, adaptive behavior and Aβ concentrations are lacking a comparative group. Finally, the high number of statistical tests carried out may increase the probability of Type-1 errors. Nonetheless, no correction to control a family-wise significance level of 0.05 has been applied in order not to increase the probability of Type-2 errors.

TABLE 6 | Correlation between cognitive and functional variables measured using Pearson's correlation coefficient.

	DMR total		ABAS total		ABAS communication skills	
	Correlation [95%-*CI*]	*p*-value	Correlation [95%-*CI*]	*p*-value	Correlation [95%-*CI*]	*p*-value
Number of correct words in 60'	−0.5 [−0.68, −0.25]	<0.001	0.32 [0.05, 0.55]	0.024	0.45 [0.2, 0.65]	0.001
Number of switches	−0.39 [−0.6, −0.12]	0.006	0.21 [−0.07, 0.46]	0.144	0.28 [0.01, 0.52]	0.046
Mean cluster size	−0.14 [−0.41, 0.14]	0.328	0.13 [−0.15, 0.4]	0.36	0.22 [−0.06, 0.47]	0.127

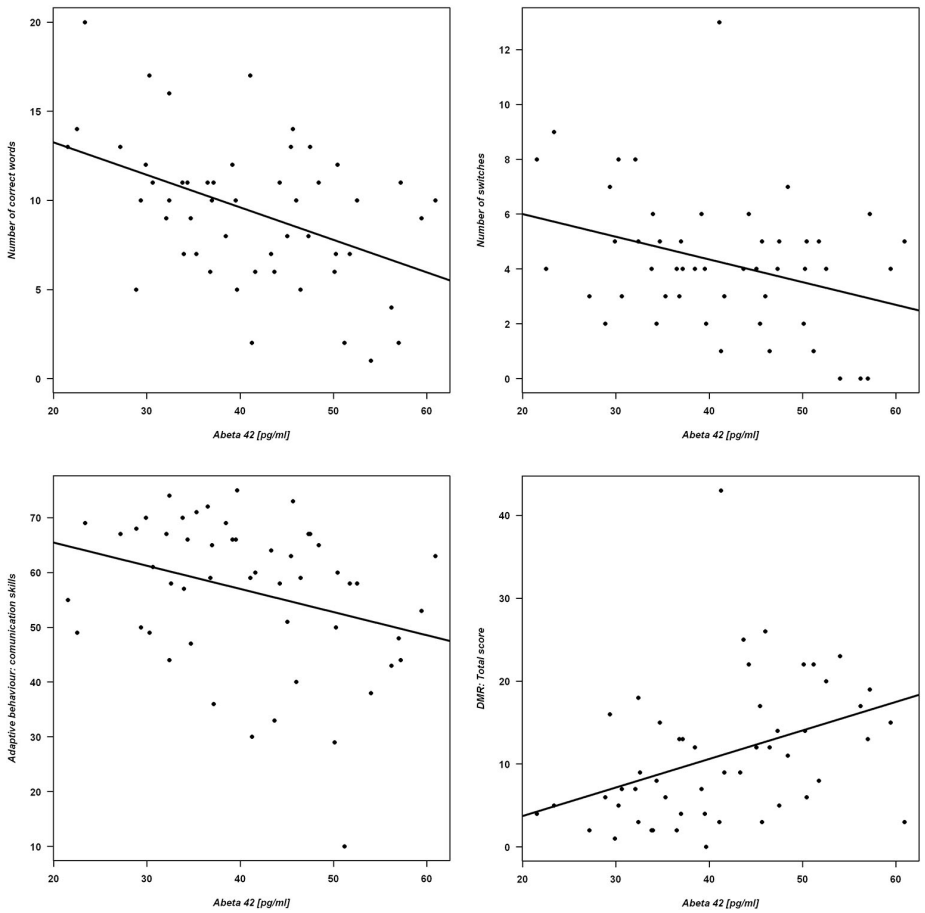

FIGURE 1 | Verbal fluency and functional measures as a function of $A\beta_{42}$ concentration. Correlations are shown for $A\beta_{42}$ and Upper panel: number of correct words (left) and number of switches (right). Lower panel: ABAS adaptive behavior (left) and DMR total score (right). The figures include the regression lines from the corresponding linear regression models.

CONCLUSION

Several studies have sought to understand the implications of changes in plasma Aβ concentrations with regard to the development of AD in DS using Mini Mental State Evaluation (MMSE), yet none has looked at the correlations between changes in concentrations and changes SVFP. Our results show an association between SVFP and early AD symptoms and plasma Aβ concentrations supporting the use of SVFT as a useful tool to detect DS subjects who are vulnerable to develop early onset AD.

Our results may be taken as a first step for further studies to find easy and fast non-invasive tools to predict the early onset of AD in DS population.

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frontiers in Behavioral Neuroscience

PERSPECTIVE
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Inter-Dependent Mechanisms Behind Cognitive Dysfunction, Vascular Biology and Alzheimer's Dementia in Down Syndrome: Multi-Faceted Roles of APP

Dean Nizetic[1,2,3]*, *Christopher L. Chen*[4,5], *Wanjin Hong*[6] *and Edward H. Koo*[7,8]

[1] *Lee Kong Chian School of Medicine, Nanyang Technological University, Singapore, Singapore,* [2] *The LonDownS Consortium, Wellcome Trust, London, UK,* [3] *The Blizard Institute, Barts and The London School of Medicine, Queen Mary University of London, London, UK,* [4] *Department of Psychological Medicine and Memory Aging and Cognition Centre, National University Health System, Singapore, Singapore,* [5] *Department of Pharmacology, National University of Singapore, Singapore, Singapore,* [6] *Agency for Science, Technology and Research (A*Star), Institute of Molecular Cell Biology, Singapore, Singapore,* [7] *Departments of Medicine and Physiology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, Singapore,* [8] *Department of Neurosciences, University of California, San Diego, San Diego, CA, USA*

People with Down syndrome (DS) virtually all develop intellectual disability (ID) of varying degree of severity, and also have a high risk of early Alzheimer's disease (AD). ID prior to the onset of dementia, and its relationship to the onset of dementia in DS is a complex phenomenon influenced by many factors, and scarcely understood. Unraveling the causative factors and modulators of these processes remains a challenge, with potential to be informative for both ID and AD, for the development of early biomarkers and/or therapeutic approaches. We review the potential relative and inter-connected roles of the chromosome 21 gene for amyloid precursor protein (APP), in both pathological conditions. Rare non-DS people with duplication of APP (dupAPP) get familial early onset AD (FEOAD) with virtually 100% penetrance and prominent cerebrovascular pathology, but don't suffer from ID before dementia onset. All of these features appear to be radically different in DS. On the other hand, rare individuals with partial trisomy 21 (T21) (with APP, but not DS-critical region in trisomy) have been described having ID. Likewise, partial T21 DS (without APP trisomy) show a range of ID, but no AD pathology. We review the multi-faceted roles of APP that might affect cognitive functioning. Given the fact that both Aβ secretion and synaptic maturation/plasticity are dependent on neuronal activity, we explore how this conflicting inter-dependency might affect cognitive pathogenesis in a dynamic way in DS, throughout the lifespan of an individual.

Keywords: Down syndrome, vascular dementia, neuron activity-dependent, cognitive dysfunction, amyloid beta-peptides, amyloid beta-protein precursor

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***Correspondence:**
Dean Nizetic
d.nizetic@ntu.edu.sg

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INTRODUCTION

Virtually all people with Down syndrome (DS) show some degree of intellectual disability (ID), due to many factors, including a certain degree of cognitive dysfunction caused by the pathobiology of trisomy 21 (T21) (Epstein, 2002; Head et al., 2012). On the other hand, T21 is the most common known genetic cause of obligatory development of pathological hallmarks of AD in the brain tissue

(Mann, 1988a; Lott and Head, 2005). This happens extremely early in virtually all people with DS (in the 30-s) (Mann, 1988a,b). The cause of this is apparently an extra copy of APP (amyloid precursor protein gene located on chromosome 21), as one individual with DS at age 74 (with no dementia, and no amyloid pathology in the brain) has been described who was born with partial chromosome 21 trisomy that did not include APP (Prasher et al., 1998). In rare families of non-DS (euploid) people, APP micro-duplications (dupAPP) are responsible for Familial Early Onset Alzheimer's Disease (FEOAD), with virtually 100% penetrance by age 60 and a median onset age of clinical dementia of 41–51, and dupAPP has so far never been seen in any human unlinked to FEOAD (Rovelet-Lecrux et al., 2006, 2007; Sleegers et al., 2006; Kasuga et al., 2009; Thonberg et al., 2011; Cohn-Hokke et al., 2012; McNaughton et al., 2012; Wallon et al., 2012). In adults with DS, the average age at onset of dementia varies greatly, with ∼25% starting extremely early (in early thirties), but a good 25–50% having a much delayed onset (compared to dupAPP), or not developing dementia at all by age >60 (Holland et al., 1998; Sekijima et al., 1998; Tyrrell et al., 2001; Coppus et al., 2006; McCarron et al., 2014). Also, cerebrovascular pathology, mainly intra-cerebral hemorrhage is a prominent symptom of dupAPP (McCarron et al., 1998; Rovelet-Lecrux et al., 2006; Sleegers et al., 2006; Kasuga et al., 2009; McNaughton et al., 2012; Wallon et al., 2012), whereas it is rarely seen in DS (Belza and Urich, 1986). This is in spite of abundant deposits of amyloid in blood vessels (congophilic angiopathy) in both conditions (see below). On the other hand, people with dupAPP largely have a normal development, intellectual and social functioning (families) prior to dementia, in contrast to people with DS.

This indicates that mechanisms are in action in DS individuals (that dupAPP individuals don't have), that cause ID, can accelerate clinical onset of dementia, as well as factors that can delay onset or protect from overt dementia and associated intra-cerebral hemorrhage.

While dupAPP patients, by definition, are seen in families exhibiting FEOAD, this implies that this is a self-selected group of people that largely lack ID prior to dementia. On the other hand, rare individuals with partial T21 (with APP, but not DS-critical chr21 region in trisomy) have been described having I.Q. ranging from ∼30 to <80, prior to dementia-prone age (Korbel et al., 2009; summarized in **Table 1**). This could be caused by an overdosed action of APP, but equally other genes included in the partial trisomy, mostly located in the "gene poor" proximal half of chromosome 21 (Groet et al., 1998). We review potential roles for APP, that go beyond the AD paradigm, and could contribute to the modulation of ID.

BEYOND ALZHEIMER'S: MULTIPLE ROLES OF APP POTENTIALLY AFFECTING COGNITIVE DYSFUNCTION IN DS (SUMMARIZED IN TABLE 2)

T21 causes synaptic plasticity defects and dendritic spine abnormalities in human brains (Marin-Padilla, 1976; Ferrer and Gullotta, 1990) as early as 19 weeks *in utero* (Weitzdoerfer et al., 2001). This pathology can be modeled using mouse models of DS (Haas et al., 2013). Dendritic spines and their plasticity are also the site of important pathology in neurodevelopmental conditions such as Rett and FraX syndromes (Troca-Marín et al., 2012; Chang et al., 2013). Their loss contributes to pathogenesis of AD and PD (McGowan et al., 2006; Schulz-Schaeffer, 2010), though specific morphological differences and causes are likely

TABLE 1 | Effects of the trisomy of APP and other segments of human chromosome 21 on development of intellectual disability (independently of dementia), and on presence of early clinical dementia.

Human genotype/ Mouse model	TRISOMY OF:			Intellectual disability independent of dementia	Early Clinical dementia <age55	References
	APP	"DS-critical Region"	Other parts of HSA21			
Down syndrome (DS)	Yes	Yes	Yes	Yes	∼60%	Summarized in Wiseman et al., 2015
DS-partial trisomy	Yes	Yes	Yes	Yes	?	Summarized in Korbel et al., 2009
DS-partial trisomy	No	Yes	Yes	Yes	No	Prasher et al., 1998
Non-DS-partial trisomy	Yes	No	Yes (large)	Yes	?	Park et al., 1987; Korbel et al., 2009
Dup-APP (majority)	Yes	No	Yes (limited)	No	>99%	Rovelet-Lecrux et al., 2006, 2007; Kasuga et al., 2009; Thonberg et al., 2011; Cohn-Hokke et al., 2012; McNaughton et al., 2012; Wallon et al., 2012
Dup-APP-only	Yes	No	No	No	>99%	Sleegers et al., 2006
Ts65Dn	Yes	Yes	Yes	Yes*	Not applicable	Reeves et al., 1995
Ts1Rhr	No	Yes	No	Yes*	Not applicable	Belichenko et al., 2009
Tc1	No	Yes	Yes	Yes*	Not applicable	O'Doherty et al., 2005; Morice et al., 2008

"?", published data are missing. "", mouse phenotypes equivalent of human ID, such as learning, memory, electrophysiological, and behavioral defects. Mouse models of trisomy 21 alone do not reproduce Alzheimers pathology in the brain, or signs of progressive neurodegenerative phenotypes (therefore "Not applicable" entry).*

TABLE 2 | An overview of a variety of processes affected by an increased dose of APP protein and/or its derivative Aβ peptides, that may contribute in DS to pathogenesis of Alzheimer's dementia or cognitive impairment (I.D.), or both.

Process affected	APP	Aβ peptides	References
Dendritic spines destruction	No	Yes	McGowan et al., 2006; Shrestha et al., 2006
Synapse loss	No	Yes	Kamenetz et al., 2003; McGowan et al., 2006; Scheff et al., 2006; Shrestha et al., 2006
GABA-ergic short-term plasticity dysfunction	Yes	No	Yang et al., 2009
Astrocytic glutamate release	Yes	Yes	Talantova et al., 2013
Extra-synaptic NMDA receptor activation	Yes	Yes	Innocent et al., 2012; Talantova et al., 2013
Receptor-mediated synaptotoxicity	No	Yes	Benilova and De Strooper, 2013
Its levels are increased by synaptic activity	No	Yes	Cirrito et al., 2005; Sullivan et al., 2013; Cheng et al., 2014
Adult hippocampal neurogenesis	Yes	No	Wang et al., 2014b
Retrograde neurotrophin signaling	Yes	No	Salehi et al., 2006
Cholinergic forebrain neuronal degeneration	Yes	No	Salehi et al., 2006
Increased levels and re-distribution of phosphorylated Tau	Yes	Yes	Israel et al., 2012; Shi et al., 2012; Moore et al., 2015
Cerebral amyloid angiopathy	Yes	Yes	Belza and Urich, 1986; McCarron et al., 1998; Rovelet-Lecrux et al., 2006, 2007; Kasuga et al., 2009; Cohn-Hokke et al., 2012; McNaughton et al., 2012; Wallon et al., 2012; Nicolas et al., 2015
Intra-cerebral hemorrhage	Yes	No	McCarron et al., 1998; Rovelet-Lecrux et al., 2006, 2007; Kasuga et al., 2009; Cohn-Hokke et al., 2012; McNaughton et al., 2012; Wallon et al., 2012

List of references is not exhaustive for all processes, publications best illustrating the point were selected.

unique to each condition. Generation and deposition of beta-amyloid peptides (Aβ40 and Aβ42) is linked with destruction of dendritic spines and synaptic loss in AD (McGowan et al., 2006; Shrestha et al., 2006). APP protein (Kamenetz et al., 2003), as well as other chromosome-21 encoded gene products, may have important functions in synaptic biology (Wang et al., 2013).

Down's syndrome causes overexpression of miR-155, a chromosome 21–encoded microRNA that negatively regulates C/EBPb, thereby reducing sorting nexin 27 (SNX27) expression and resulting in synaptic dysfunction (Wang et al., 2013). SNX27 is a novel activity-dependent signaling molecule that has the ability to decode the Ras signal and transduce the plasticity stimuli to the delivery of postsynaptic AMPA receptors (Loo et al., 2014). So, SNX27 signaling is also activity-dependent: the more neuronal activity, the bigger chances of seeing pathology due to inability to raise sufficient SNX27 levels. On the other hand, SNX27 acts as a γ-secretase interaction partner to promote dissociation of the γ-secretase complex, thus decreasing its proteolytic activity, thereby reducing the generation of all Aβ peptides (Wang et al., 2014a). The effects of the apparent reduction of SNX27 in DS would therefore be expected to further increase Aβ levels, (by lessening the dissociation of the γ-secretase complex), and therefore worsen AD-pathology. However, the end results of this on AD pathogenesis are far from simple, and need to be carefully further investigated. While inhibition of γ-secretase activity might reduce the levels of neurotoxic Aβ42, intriguingly, very recent results on hiPSC-derived neurons show that chemical inhibition of γ-secretase activity in T21 neurons dramatically increased levels of Tau, the principal constituent of neurofibrillary tangles. This could actually have pro-dementia effects (Moore et al., 2015). The picture is even more intriguing, as the same study found that addition of a γ-secretase modulator (GSM), E2012, decreased Tau levels in T21 neurons (that showed an otherwise increased Tau levels; Moore et al., 2015).

Synaptic dysfunction is an early feature of AD, likely much before significant Aβ deposition (Arendt, 2009). There is a line of thought that AD neurodegenerative processes begin by alterations in synapse function/structure leading to synapse loss, prior to significant neuronal loss. Consistent with this, disturbance in synaptic integrity is detected in patients with mild cognitive impairment (MCI), which is sometimes perceived as an early stage of AD (Scheff et al., 2006). Loss of synaptic markers is a predictor of disease progression in AD (Selkoe, 2002). Aβ, generated from proteolytic processing of APP, has been shown to disrupt synapses (Kamenetz et al., 2003; Scheff et al., 2006; Shrestha et al., 2006) and, conversely, synaptic activity is an important factor regulating Aβ levels (Cirrito et al., 2005; Sullivan et al., 2013; Cheng et al., 2014). Aβ was also found to induce astrocytic glutamate release, extra-synaptic NMDA receptor activation, and synaptic loss (Talantova et al., 2013).

APP and Aβ have other, direct and indirect roles in functioning of synapses. Overexpression of APP affects $Ca_v1.2$ L-type calcium channel levels and through this influence GABAergic short-term plasticity (Yang et al., 2009). APP may contribute to postsynaptic mechanisms via the regulation of the surface trafficking of excitatory N-methyl-D-aspartate (NMDA) receptors (Innocent et al., 2012). Aβ was also shown binding to a number of receptors embedded in neuronal plasma membrane, (such as PrP, EphB2, FcγRIIb, and PirB), potentially contributing to receptor-mediated synaptotoxic pathways (Benilova and De Strooper, 2013).

There are also compelling evidence for alterations in synaptic function/plasticity in DS mouse models. For instance, altered excitatory/inhibitory balance is known to modify synaptic plasticity in DS mouse models (Kleschevnikov et al., 2004;

Souchet et al., 2014). Importantly, increased APP expression in mouse models of DS has other deleterious effects that could be linked with neurodevelopmental milestone delays (and resulting ID): APP controls adult hippocampal neurogenesis, maintaining the tone of action of inhibitory GABAergic interneurons (Wang et al., 2014b). Interestingly, in the Ts65Dn partial trisomy 16 mice, defects in retrograde neurotrophin signaling and cholinergic forebrain neuronal degeneration are specifically related to extra APP gene dosage (Salehi et al., 2006). Similarly, endosomal abnormalities in the form of enlarged organelles which are characteristic changes in brains of both AD and DS individuals and also a consequence of increased APP expression (Cataldo et al., 2003). Whether these alterations are due to elevated APP or Aβ, or both, is not clear although there are suggestions from cultured neurons that endosomal dysfunction may be Aβ-independent (Jiang et al., 2010). Also, in lymphoblastoid cell lines carrying amyloid precursor protein (APP) microduplications causing autosomal dominant EOAD, enlarged endosomes were absent, suggesting that APP overexpression alone is not involved in the modification of early endosomes, but overexpression of other chromosome 21 genes plays an important role (Cossec et al., 2012). Finally, APP whole protein was also shown binding to Aβ, adding to the complexity of the potentially pathological interactions (Lorenzo et al., 2000).

WHY ARE VASCULAR AND MIXED DEMENTIA NOT PREVALENT IN DS?

Mixed dementia (MD)—defined as the coexistence of Alzheimer's disease (AD) and cerebrovascular disease (CVD) (Rockwood, 2003) has been identified as one of the most common subtypes of dementia by autopsy-based epidemiological studies (Skoog et al., 1993; Snowdon et al., 1997). The presence and degree of CVD modulates the cognitive picture of AD (Dong et al., 2013). Neuropathological studies have shown that infarcts increase the odds of dementia in patients with equivalent AD burden by adding to the deleterious effects of AD pathology (Schneider et al., 2007). While most common in hypertensive individuals, intracerebral hemorrhage has been reported in 20–50% of APP-Dup cases (Rovelet-Lecrux et al., 2006, 2007; Kasuga et al., 2009; Cohn-Hokke et al., 2012; McNaughton et al., 2012; Wallon et al., 2012), whereas individuals with DS are generally protected from this pathology. Reasons for this protection in DS individuals are unknown, but are potentially very important. Both dupAPP and DS show severe cerebral amyloid angiopathy (CAA) (Belza and Urich, 1986; McCarron et al., 1998; Rovelet-Lecrux et al., 2006, 2007; Kasuga et al., 2009; Cohn-Hokke et al., 2012; McNaughton et al., 2012; Wallon et al., 2012) which renders blood vessels more susceptible to vessel wall breakdown and subsequent hemorrhage. It remains to be answered whether more general disturbances of vascular physiology seen in DS (but not in dupAPP) have anything to do with the apparent protection from vascular dementia and higher frequency of cerebral hemorrhages. Several effects caused by T21 have been described that could affect vascular biology (Vis et al., 2009; Draheim et al., 2010). T21 has a powerful effect on inhibition of angiogenesis, and a reduced response to pro-angiogenic cytokines, such as VEGF-A (Arron et al., 2006). This biological feature of T21 is one of the explanations for the reduced incidence of solid tissue tumors in DS (Baek et al., 2009; Yang and Reeves, 2011; Nizetic and Groet, 2012). This is attributed to at least 7 genes on HSA21, and full mechanisms are not completely understood (Zorick et al., 2001; Ryeom et al., 2003; Arron et al., 2006; Baek et al., 2009; Reynolds et al., 2010; Yang and Reeves, 2011; Nizetic and Groet, 2012).

As regards atherosclerotic cardiovascular disease (Vis et al., 2009), although abnormalities in lipid metabolism, which are associated with high risk of premature atherosclerosis in the general population (increased triglycerides, decreased HDL), are frequently seen in patients with DS (Dörner et al., 1984; Bocconi et al., 1997; Corsi et al., 2005), and despite reduced physical activity and high rates of obesity (de Winter et al., 2012), atherosclerosis and coronary artery disease related mortality is surprisingly low (Baird and Sadovnick, 1988; Corsi et al., 2005; Lott and Head, 2005), a finding that led some authors to conclude that DS may represent an atheroma-free model of disease (Murdoch et al., 1977). Interestingly, hyper-triglyceridaemia, increased obesity and low exercise rates are common in adults with DS (Haas et al., 2013), and high cholesterol levels have been associated with risk of developing dementia (Chang et al., 2013). However, some cardiovascular risk factors, including hypertension, atherosclerosis, and smoking (McGowan et al., 2006; Troca-Marín et al., 2012) that are thought to contribute to the development of dementia in the general population (Schulz-Schaeffer, 2010), are lower among adults with DS.

Other DS-specific biological features may also play a role. While DS neurons show a severe down-regulation of mitochondrial function (Busciglio and Yankner, 1995; Roat et al., 2007; Murray et al., 2015), multiple studies indicate that reducing mitochondrial function can protect against aging and age-associated diseases (Trifunovic and Ventura, 2014). DS cells demonstrate adaptive down-regulation of mitochondrial function for survival under increased ROS conditions (Helguera et al., 2013).

IS INTENSIFIED NEURONAL ACTIVITY GOOD OR BAD FOR LIFE-LONG DS COGNITIVE FUNCTIONING?

Recent work on mouse models has shown that hyperactivity of GABAergic interneurons in mouse models of DS over-inhibits hippocampal cortical excitatory neurons (Fernandez et al., 2007; Kleschevnikov et al., 2011). This has resulted in the first clinical trials in adults with DS for the improvement of cognitive functions, for the cognitive enhancement with GABA-α5 inverse agonists (Martínez-Cué et al., 2014). So, increased activity of one type of neurons is causing a decreased activity of another type of neurons in DS.

However, by increasing the neuronal activity of hippocampal cortical excitatory neurons, in theory, we also increase Aβ production and release. In fact, it has been demonstrated that both Aβ secretion, and synaptic plasticity are dependent on

neuronal activity (Kamenetz et al., 2003; Cirrito et al., 2005; Scheff et al., 2006; Shrestha et al., 2006; Cheng et al., 2014; Lundgren et al., 2014). It remains unclear though whether inhibitory neurons have as much APP as excitatory neurons, or, whether over activity of inhibitory vs. excitatory neurons drive more Aβ generation. A proteolytic fragment (p25) of the cdk5-activator is generated as a function of neuronal activity, and it regulates synaptic plasticity and Aβ-induced cognitive impairment (Seo et al., 2014). Aβ-generation, endosomal trafficking and secretion (Wu et al., 2011; Sullivan et al., 2013; Lundgren et al., 2014) and Aβ-dependent Tau translocation to excitatory synapses (Frandemiche et al., 2014) are all neuronal activity-dependent. On the other hand, synaptic plasticity and synaptic maturation have also been shown to be neuronal activity-dependent processes (Fukazawa et al., 2003; Segal, 2005, 2010; Bosch and Hayashi, 2012; Heimer-McGinn et al., 2013; Ramiro-Cortés and Israely, 2013).

This seemingly controversial roles of neuronal activity level in DS pathology have also repercussions when it comes to therapeutic management approaches: sleep deprivation contributes to increased Aβ generation/secretion (Kang et al., 2009) as well as reduced clearance (Xie et al., 2013), and sleep deprivation in DS was shown to affect cognitive function (Brooks et al., 2015). On the other hand, deep brain stimulation was proposed as one of the intervention approaches to ameliorate cognitive dysfunction in AD (Boggio et al., 2011). More research is needed in this direction, as clearly opposing consequences could be reached, if the approaches are not better understood, and accordingly fine-tuned.

In conclusion, there are clearly many open questions on the inter-relation between pathogenic processes that affect neuronal development, synaptic plasticity, neuronal aging and longevity, and AD in DS. We have not had the space or scope in this mini-review to mention the potential modulating action of all other chromosome 21 genes that might influence this process, besides APP. Much more research is needed using high-resolution dissection of individual chr21 gene contributions using mouse models and human iPSC modeling. Such efforts should be coordinated and inter-disciplinary, including clinical dementia and cognitive assessments, and imaging studies.

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Assessment of Cognitive Scales to Examine Memory, Executive Function and Language in Individuals with Down Syndrome: Implications of a 6-month Observational Study

Xavier Liogier d'Ardhuy[1†], Jamie O. Edgin[2†], Charles Bouis[3], Susana de Sola[4], Celia Goeldner[1], Priya Kishnani[5], Jana Nöldeke[1], Sydney Rice[6], Silvia Sacco[3], Lisa Squassante[7], Gail Spiridigliozzi[8], Jeannie Visootsak[9], James Heller[10‡] and Omar Khwaja[11]*

[1] F. Hoffmann-La Roche, Roche Pharma Research and Early Development, Neuroscience, Roche Innovation Center Basel, Basel, Switzerland, [2] Department of Psychology, University of Arizona, Tucson, AZ, USA, [3] Research Department, Institut Jérôme Lejeune, Paris, France, [4] Cellular and Systems Neurobiology Research Group, Human Pharmacology and Clinical Neurosciences Research Group-Neurosciences Program, Systems Biology Program, Centre for Genomic Regulation, Hospital del Mar Medical Research Institute, Barcelona, Spain, [5] Medical Genetics, Duke University Medical Center, Durham, NC, USA, [6] Department of Pediatrics, University of Arizona, Tucson, AZ, USA, [7] F. Hoffmann-La Roche, Biostatistics, Basel, Switzerland, [8] Department of Pediatrics, Duke University Medical Center, Durham, NC, USA, [9] F. Hoffmann-La Roche, Roche Pharma Research and Early Development, Neuroscience and Rare Diseases, Roche Innovation Center New York, New York, NY, USA, [10] Formerly of Duke University Medical Center, Durham, NC, USA, [11] F. Hoffmann-La Roche, Roche Pharma Research and Early Development, Rare Diseases, Roche Innovation Center Basel, Basel, Switzerland

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Roger H. Reeves,
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H. Craig Heller,
Stanford University, USA
Alexander M. Kleschevnikov,
University of California, San Diego, USA

***Correspondence:**
Xavier Liogier d'Ardhuy
xavier.liogier_dardhuy@roche.com

[†] These authors have contributed equally to this work.
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Down syndrome (DS) is the most commonly identifiable genetic form of intellectual disability. Individuals with DS have considerable deficits in intellectual functioning (i.e., low intellectual quotient, delayed learning and/or impaired language development) and adaptive behavior. Previous pharmacological studies in this population have been limited by a lack of appropriate endpoints that accurately measured change in cognitive and functional abilities. Therefore, the current longitudinal observational study assessed the suitability and reliability of existing cognitive scales to determine which tools would be the most effective in future interventional clinical studies. Subtests of the Repeatable Battery for the Assessment of Neuropsychological Status (RBANS), Cambridge Neuropsychological Test Automated Battery (CANTAB), and Clinical Evaluation of Language Fundamentals-Preschool-2 (CELF-P-2), and the Observer Memory Questionnaire-Parent Form (OMQ-PF), Behavior Rating Inventory of Executive Function®–Preschool Version (BRIEF-P) and Leiter International Performance Scale-Revised were assessed. The results reported here have contributed to the optimization of trial design and endpoint selection for the Phase 2 study of a new selective negative allosteric modulator of the $GABA_A$ receptor α5-subtype (Basmisanil), and can be applied to other studies in the DS population.

Keywords: Down syndrome, outcome measure, clinical trial, cognition, language

INTRODUCTION

Down syndrome (DS) is the most common chromosomal cause of intellectual disability (ID). Each year approximately 6000 babies are born in the United States with DS, which is equivalent to 1 in 700 babies (Parker et al., 2010). Worldwide the estimated incidence is approximately 1 in 1000–1100 (World Health Organization (WHO), 2015). DS is characterized by substantial limitations in intellectual functioning (i.e., low intellectual quotient (IQ), delayed learning and/or impaired language development) and adaptive behavior. Studies have revealed a specific neuropsychological profile for this population—individuals typically have an average IQ below 70 (Chapman and Hesketh, 2000; Gioia et al., 2000) and weaknesses consistently associated with associative and verbal working memory (Jarrold et al., 2006, 2008; Silverman, 2007), episodic memory and explicit long-term memory (Carlesimo et al., 1997; Vicari, 2001), expressive language (Miller, 1998), and executive function (Lanfranchi et al., 2010), whereas relative strengths have been observed in visuospatial tasks and implicit long-term memory (Edgin et al., 2010b). Although, IQ levels vary in individuals with DS, most individuals function in the mild to moderate range of ID (Centers for Disease Control Prevention, 2015; Centers for Medicare Medicaid Services, 2014). Of note, as the rate of cognitive development progressively becomes slower over the childhood years in relation to typically developing peers, a decline in IQ scores over the childhood years is also observed (Carr, 1995).

Differences in brain structure and function are already apparent in early infancy in individuals with DS (Nadel, 2003; Edgin et al., 2015), with clear alterations in hippocampus (e.g., altered microarchitecture of pyramidal cells), prefrontal cortex (reduced volume), and cerebellum (e.g., hypoplasia) apparent pre- and post-natally (Pennington et al., 2003; Lott and Dierssen, 2010). Furthermore, structural and volumetric magnetic resonance imaging (MRI) studies have shown that individuals with DS have a smaller intracranial volume than their typically developing peers, with the most profound differences observed in the frontal lobes, cerebellum, and brainstem (Kesslak et al., 1994; Raz et al., 1995; Aylward et al., 1999). Other studies have also shown that smaller volumes are observed in the temporal lobe, including the hippocampal region (Schmidt-Sidor et al., 1990; Pinter et al., 2001) which is known to affect a range of cognitive functions. As individuals with DS approach early adulthood, some are at particular risk for the early development of Alzheimer's disease (Zigman et al., 2008). The prevalence of dementia in DS increases over 45 years of age, with upwards of 75% having dementia over 65 years (Lott and Dierssen, 2010), although neuropathological and neurochemical changes have been observed as early as fetal development (Bahn et al., 2002; de Sola et al., 2015).

Recent advancements in our understanding of the underlying mechanisms of cognitive dysfunction in DS suggest an imbalance between excitatory and inhibitory neurotransmission. γ-Aminobutyric acid (GABA) neurotransmission is the major inhibitory system in the mature brain. Reducing GABA-mediated inhibition by limiting $GABA_A$ receptor activity has shown beneficial effects on hippocampal synaptic plasticity as well as learning and memory deficits in the Ts65Dn mouse model of DS (Kleschevnikov et al., 2004; Fernandez et al., 2007; Colas et al., 2013; Martínez-Cué et al., 2013; Potier et al., 2014). A negative allosteric modulator of the $GABA_A$ α5-containing receptor subtype (Basmisanil) is currently under investigation in young adults with DS (ClinicalTrials.gov identifier: NCT02024789).

Previous pharmaceutical trials in DS have noted that studies are often limited by a lack of endpoints that accurately captured cognitive and functional changes (Heller et al., 2006). Thus, it is important to assess the suitability and reliability of existing tools that measure cognitive function in a longitudinal observational study to determine which measures may be most effective in the context of a pharmacological clinical trial. Specifically, clinical trials require measures that can be repeatedly and reliably administered across international sites, to participants of a defined age range, and that do not exhibit large practice, floor, or ceiling effects.

The recently published TESDAD battery includes neurocognitive tests and scales, but no test-retest analysis or evaluation of potential practice effect are currently available (de Sola et al., 2015). Edgin et al. also reported the development of the Arizona Cognitive Test Battery (ACTB) based on the Cambridge Neuropsychological Test Automated Battery (CANTAB) and other available tools (Edgin et al., 2010a). The ACTB was designed based on historical findings of performance deficits in domains, and tasks that had been repeatedly shown to be more difficult for those with DS (Pennington et al., 2003; Edgin et al., 2010a, 2014; Lee et al., 2011). The ACTB validation suggested that neuropsychological measures could be administered to a large sample of individuals with DS ($n = 74$) with low floor effects and good preliminary estimates of test-retest reliability (albeit in a small subsample). This battery could have been used in our clinical trials; however, based on the mechanism of action of Basmisanil, some of the tests may be more relevant than others (e.g., hippocampal or prefrontal tests vs. cerebellar function tests). Therefore, alternative scales were chosen for analysis in this study. Furthermore, most measurement validation studies have been limited in their ability to ascertain the reliability of endpoint measures within the retesting time frame and frequency required to determine how the measures perform in a clinical trial context. Given the frequency of new clinical investigations in this population, more measurement development and validation is urgently required, leading us to report on these data to assist the broader community with study design in the future. Furthermore, the National Institutes of Health (NIH) Research Plan on Down Syndrome, which was revised in 2014, reports on the need to study clinical and behavioral treatments and interventions for DS, with part of this plan noting the importance for reliable and valid endpoint assessments to measure the efficacy of these treatments (U.S. Department of Health Human Services National Institutes of Health, 2014).

OBJECTIVES

Given this background, the primary objective of this non-pharmacological study (BP25612; ClinicalTrials.gov identifier:

NCT01580384) was to investigate the suitability (i.e., number of participants completing the tests, floor/ceiling effects, and potential learning effect) of selected neurocognitive tests in a 6-month longitudinal and multinational setting for the measurement of cognitive function in individuals with DS. Subtests of the Repeatable Battery for the Assessment of Neuropsychological Status (RBANS) (Randolph et al., 1998), subtests of CANTAB (Cantab Research Suite, 2015), subtests from the Clinical Evaluation of Language Fundamentals-Preschool-2 (CELF-P-2) (Pearson, 2004), the Observer Memory Questionnaire-Parent Form (OMQ-PF) (Gonzalez et al., 2008), and the Behavior Rating Inventory of Executive Function®–Preschool Version (BRIEF-P) (Gioia et al., 2000) were used to assess immediate and delayed memory, language, and executive function. Secondary objectives were to assess the test-retest reliability of these measures over 6 months and to explore the influence of age (adolescents vs. adults) and non-verbal IQ level, as measured by the Leiter International Performance Scale-Revised (Leiter-R) (Roid and Miller, 1997).

Part of the results from this study were previously presented at the 2014 American Association of Intellectual and Developmental Disabilities (AAIDD) Annual Meeting (del Valle Rubido et al., 2014), as well as at the 2013 Cognition in Down Syndrome Workshop (Liogier d'Ardhuy et al., 2013). Results from the assessments using the Vineland Adaptive Behavior Scales-II (VABS-II) and the Clinician Global Impression of Severity (CGI-S) and Improvement (CGI-I) scales will be reported separately.

METHODS

This was a 6-month (24–27 weeks) observational, non-pharmacological, longitudinal, multicenter (11 sites), multinational study in adolescents (12–17 years) and adults (18–30 years) with DS conducted between February 2012 and January 2014. The study was conducted in the United States, United Kingdom, Spain, France, Italy, Canada, and Argentina. Overall 90 participants (equally split between adolescents and adults) were planned to be enrolled and randomized into three different schedules of assessments (i.e., A, B, and C; C contained a smaller number of tests and visits). In order to include all of the planned assessments and keep the duration within the desired 90-min testing period for each study visit, three schedules of assessments were implemented. A 15–25 min break was planned after 45 min of testing and an additional break could be added before starting the last exercise (RBANS) if requested or deemed necessary by the rater. Randomization was stratified by age group to have a balanced number of sequences of assessments between adolescents and adults.

The current study was conducted for 6 months to reflect the clinical trial design of the ongoing Phase 2 study. Participants who met the inclusion criteria (below) received testing at the baseline visit, 4 weeks and 24 weeks later when randomized to schedule A or B or received testing at the baseline visit and at 24 weeks when randomized to schedule C (**Table 1**). These schedules resulted in a common data set that was administered to at least 60 participants. The total duration of the study for each participant was between 24 and 27 weeks.

TABLE 1 | Number of participants per randomization schedule and total number of subjects evaluated per task.

Scale	Subscale	Schedule			Total number of participants
		A	B	C	
Leiter-R		30	30	30	90
CANTAB	SSP	30	30		60
CELF-P-2		30	30	30	90
RBANS	List learning	30	30	30	90
	Story memory	30	30	30	90
	Picture naming	30	30	30	90
	Semantic fluency	30	30	30	90
OMQ-PF		30	30	30	90
BRIEF-P		30	30		60

Randomization was stratified by age, with an equal number of participants in the 12–17 and 18–30 years age groups. For schedules A and B assessments were done at baseline, week 4 and week 24. For schedule C assessments were done at baseline and week 24 only.

Study Population

Male and female adolescents (12–17 years) and adults (18–30 years) with a diagnosis of DS were included in the study if they met all of the following criteria: parent/caregiver was able to speak and understand the local language, to accompany the participant to all clinic visits, and to provide information about the participant's behavior and daily functioning. Also, the participant's speech was understandable to the examiner; at screening the participant attempted to perform the neuropsychological tests; stable treatment for at least 8 weeks prior to screening if he/she had a generalized anxiety disorder, major depressive disorder, autism spectrum disorder, attention-deficit/hyperactivity disorder, and recent laboratory tests confirming euthyroid (serum free thyroxine [FT4] and thyroid stimulating hormone [TSH]) and normoglycemic (serum glucose) status (within 12 months prior to screening visit, with or without treatment). Individuals were not included if they met any of the following criteria: diagnosed with axis I and II psychiatric disorders, except those mentioned above; exhibited significant suicidal risk; could not comply with protocol or perform the outcome measures due to hearing or visual impairment; had evidence of dementia; had thyroid dysfunction or diabetes not adequately controlled at least 8 weeks prior to randomization; or abused alcohol and/or other substances.

Written informed consent was obtained from the parents/caregivers and assent from the participants prior to participation in the study. The study was conducted in accordance with the principles of the Declaration of Helsinki and Good Clinical Practice (GCP), and all required approvals were obtained from the appropriate independent ethics committee (IEC)/institutional review board (IRB) prior to the start of the study.

Concomitant Medication

Psychotropic agents that would likely interfere with any of the assessments could not be initiated or changed during the study period. This included antidepressants (e.g., selective serotonin reuptake inhibitors [SSRIs], serotonin and norepinephrine reuptake inhibitors [SNRIs], norepinephrine-dopamine reuptake inhibitors such as bupropion, and serotonin-norepinephrine reuptake inhibitors such as the tricyclic antidepressants), antipsychotics, benzodiazepines and hypnotics, acetylcholinesterase inhibitors, GABA agonists (e.g., tiagabine, vigabatrin, and baclofen), and glutamatergic drugs (e.g., riluzole, topiramate, memantine, and lamotrigine).

Procedures

Selected raters for the cognitive assessments/rating scales were provided with instructions and comprehensive training on scale administration prior to the start of the study. Whenever possible, for each participant the same rater/caregiver consistently administered/completed the rating scales across study visits.

The assessments were completed in a prespecified and consistent order to maximize standardization across sites and participants.

Scales Selected to Measure Cognitive Skills

The Leiter International Performance Scale-revised (Leiter-R) (Roid and Miller, 1997)

Leiter-R, a non-verbal intelligence test, was individually administered to all participants. Two reasoning subtests (Sequential Order and Repeated Patterns) and two visualization subtests (Figure Ground and Form Completion) were administered to derive a non-verbal IQ.

Repeatable Battery for the Assessment of Neuropsychological Status (RBANS) (Randolph et al., 1998)

The RBANS was individually administered and used to measure cognitive changes over time. Four subtests of the full battery of 12 subtests were used in this study to assess immediate memory (List Learning and Story Memory), as well as language capacities (i.e., Picture Naming and Semantic Fluency). The RBANS was chosen because it has been used in clinical trials investigations (Duff et al., 2010; Hobson et al., 2010) and provides four alternate forms. Alternate forms were used on each study day. The raw score on each of these scales was used for analysis.

Cambridge Neuropsychological Test Automated Battery (CANTAB) (Cantab Research suite, 2015)

The CANTAB is a computerized battery of neuropsychological tests carried out by the participant under the supervision of qualified personnel. The Spatial Span (SSP) subtest was used in this study to assess working memory capacities; it is considered a visuospatial analog of a digit span test in which a random array of boxes on a screen change color in a particular sequence. The participant's response was given by recalling the test pattern in forward or reverse order.

Observer Memory Questionnaire-parent Form (OMQ-PF) (Gonzalez et al., 2008)

The OMQ-PF is a 27-item questionnaire designed to ascertain the perceptions of parents/caregivers about the participant's daily memory function. It has been previously validated in children with temporal lobe epilepsy and memory impairment (Gonzalez et al., 2008). Items were rated on a 5-point Likert scale (1- strongly agree to 5- strongly disagree OR 1- never to 5- always).

Behavior Rating Inventory of Executive Function®–Preschool Version (BRIEF-P) (Gioia et al., 2000)

The BRIEF-P was completed by the parent/caregiver and measured the participant's everyday skills associated with executive function (i.e., Inhibit, Working Memory, Plan/Organize, and the Global Executive Composite [GEC]). This scale has been used in a number of investigations of DS, where it demonstrated a unique pattern of strengths and weaknesses, including deficits in parent's ratings of working memory and planning, but not in inhibition or emotional control (Lee et al., 2011).

Clinical Evaluation of Language Fundamentals-Preschool-2 (CELF-P-2) (Pearson, 2004)

The CELF-P-2 consists of a variety of subtests used to evaluate the language skills of preschool-aged children (3–6 years). The Word Classes subtest was used to evaluate the participant's ability to understand and express relationships between semantically related words. Raw scores from the receptive and expressive scales of this subtest were used in the analyses.

The CELF-P-2, RBANS, and OMQ-PF were translated into French, Italian, and Spanish by a process that included forward translation, back translation, and concept validation. Rater instructions for the Leiter-R and CANTAB were also translated. The BRIEF-P was already available in various languages and did not require translation for use in this study.

Statistical Methods

For the assessments with a minimum of 60 participants, a Mixed Model Repeated Measurements (MMRM) analysis was applied with visit-time as repeat factor; subject as subject-effect; gender, language and age as class factors; age by visit-time as interaction; and baseline IQ as continuous covariate. Estimates of the mean differences between age groups, genders and visits (6 months vs. baseline), and the estimate of the slope (β) over IQ were derived.

Measurements of between-subject variability and residual variability as well as of correlation between repeated assessments within the same subjects were extracted from the mixed model. As a measure of test-retest reliability, Intraclass Correlation Coefficient (ICC) was derived per each age group between visits (6 months vs. baseline). An ICC was considered poor, fair, good, and very good when values were <0.40, 0.40–0.59, 0.60–0.75, and >0.75, respectively (Cicchetti and Sparrow, 1981; Oremus et al., 2012). Analyses of correlation at baseline were performed between RBANS List Learning and both CELF-P-2 Expressive

and OMQ-PF scores, and between CANTAB SSP reverse and BRIEF-P scores (i.e., GEC and Working Memory subdomains).

All derived *p*-values were not controlled for multiple comparisons and should be interpreted as an aid to gauge the magnitude of estimated differences.

RESULTS

Study Population

A total of 94 participants were screened, 90 were randomized (49 adolescents 12–17 years; 41 young adults 18–30 years), and 89 completed the study; the participant who did not complete the study was lost to follow up. **Table 2** shows the study demographics. The mean age for the adolescent and adult groups was 15 years and 23 years, respectively. The adult group was well balanced for gender (51% female, 49% male), whereas slightly more males were enrolled in the adolescent group (59%). No procedure-related adverse events (e.g., fatigue or tiredness) were recorded in any participants.

Neurocognitive Assessments

The baseline IQ scores are shown in **Table 2**. The mean IQ scores were similar between age groups (adolescents 42 ± 7; adults 39 ± 6), although 22% of adolescents and 61% of adults performed at the floor (36) of the test (**Table 3**).

Memory Assessments

RBANS (List Learning and Story Memory)

The List Learning baseline scores followed a relatively normal distribution, ranging from 0 to 32, over a maximum possible score of 40, with means of 11.8 (standard deviation [SD] 7.5) and 13.8 (SD 8.2) for the adolescents and adults, respectively (**Table 4**). Very few participants had a score of zero in this task (**Table 3**; 4 and 7% for adolescents and adults, respectively). However, 24% of adolescents and 12% of adults had very low scores (≤4). The average reference List Learning scores for typically developing individuals aged 20–39 years is approximately 30 (Randolph, 2006). Overall, adults had statistically higher List Learning scores than adolescents (age, $p = 0.035$; **Table 5**). The adolescents showed improvement (+2.3 ± 5.6) over the 6-month period, whereas the adults did not, as captured by the close to significant time x age interaction. The IQ scores were significantly related to the List Learning scores ($p < 0.001$; **Table 5**).

Overall, the Story Memory scores ranged from 0 to 21 out of a maximum possible score of 24 with means of 5.6 (SD 4.1) and 6.0 (SD 5.2) for the adults and adolescents, respectively (**Table 4**). The distribution was skewed toward the lower scores, illustrating a floor effect. This was particularly evident in the adolescent group, with 22% obtaining a score of 0 at baseline, reflecting the difficulty of this subtest for this population. However, on average, both age groups performed equally in the Story Memory subtest ($p = 0.250$; **Table 4**). Adolescents scores decreased on average over the 6-month period (−1.6 ± 3.5 SD), whereas adult scores did not change over time (time × age, $p = 0.030$; **Table 5**). IQ scores were significantly related to the Story Memory scores ($p = 0.001$; **Table 5**).

TABLE 2 | Study demographics.

	12–17 years	18–30 years
N	49	41
Females	20 (41%)	21 (51%)
Males	29 (59%)	20 (49%)
AGE		
Mean ± SD	14.5 ± 1.6	22.7 ± 3.4
Median	15	22
Range	12–17	18–30
IQ (LEITER-R)		
Mean ± SD	41.6 ± 7.1	39.0 ± 6.0
Mean (F/M)	39.9/42.7	40.4/37.6
Range	36–80	36–65
COUNTRY (*N*)		
Argentina	7	3
Canada	2	6
France	17	6
Italy	6	5
Spain	11	6
UK	0	2
US	6	13

OMQ-PF (Daily Memory)

The baseline distributions of total raw scores for both age groups appeared normal, ranging from 61 to 124 (reference for typically developing children 5–16 years of age, 107). There was no significant difference in the observed memory scores between age groups (6.09, $p = 0.075$) or visits (0.21, $p = 0.824$; **Table 5**). IQ level did not predict perceived daily memory scores. The observed memory score correlated with the RBANS List Learning score across ages ($r = 0.33$, $p < 0.01$), demonstrating concurrent validity with a direct memory assessment.

Executive Function Assessments

CANTAB (Spatial Span)

For the forward span length, the baseline distribution was normal in both age groups and no floor effect was observed (**Table 5**). On the other hand, in the reverse task, 24% of the adolescents and 22% of the adults scored 0. On average, adults had significantly greater reverse span length (+0.77, $p = 0.019$; **Table 5**), whereas no difference was observed between age groups for the forward span performance (age, $p = 0.095$). Forward and reverse span lengths were stable over time (age × time 0.814 and 0.435, respectively; **Table 5**). IQ was related to both forward ($p < 0.001$) and reverse ($p = 0.001$) span length (**Table 5**).

BRIEF-P

At baseline, the BRIEF-P GEC scores in the adolescent group were normally distributed, whereas the adult group peaked at lower values (better). Adults had statistically lower mean BRIEF-P GEC scores compared with adolescent (−13.42, $p = 0.011$), indicating higher perceived executive functioning in this age group. GEC scores were stable across visits (time, $p = 0.291$).

TABLE 3 | Test-retest reliability (ICC) between baseline and 6 months and floor effect at baseline.

		Leiter-R	BRIEF-P (composite)	CELF-P-2 (expressive)	CELF-P-2 (receptive)	CANTAB SSP length FW	CANTAB SSP length REV	OMQ-PF	RBANS Picture Naming	RBANS Semantic Fluency	RBANS List Learning	RBANS Story Memory
Adolescents	ICC	NA	**0.78**	**0.71**	**0.63**	**0.67**	0.53	**0.76**	0.50	0.59	**0.69**	**0.69**
	Floor*	11/49 (22%)	NA	7/49 (14%)	1/49 (2%)	1/34 (3%)	8/34 (24%)	NA	5/49 (10%)	3/49 (6%)	2/49 (4%)	11/49 (22%)
Adults	ICC	NA	**0.77**	**0.69**	**0.68**	0.55	0.40	**0.90**	0.53	**0.73**	**0.64**	**0.67**
	Floor*	25/41 (61%)	NA	3/41 (7%)	1/41 (2%)	0 (0%)	6/27 (22%)	NA	2/41 (5%)	3/41 (7%)	3/41 (7%)	5/41 (12%)

**Floor: number of subjects at the lowest possible value of the assessment over the total number of subjects assessed. Bold values correspond to ICC ≥ 0.60 (good).*

TABLE 4 | Mean scores and standard deviations at baseline and 6 months for each scale.

Mean ± SD (*N*)		12–17 years	18–30 years
RBANS list learning	Baseline	11.8 ± 7.5 (49)	13.8 ± 8.2 (41)
	6 Months	14.1 ± 7.3 (49)	13.8 ± 7.0 (39)
RBANS story memory	Baseline	6.0 ± 5.2 (49)	5.6 ± 4.1 (41)
	6 Months	4.4 ± 3.4 (49)	5.5 ± 4.3 (39)
RBANS picture naming	Baseline	6.3 ± 2.3 (49)	6.4 ± 2.1 (41)
	6 Months	6.4 ± 2.4 (49)	6.7 ± 2.7 (39)
RBANS semantic fluency	Baseline	8.1 ± 5.1 (41)	7.2 ± 3.7 (49)
	6 Months	6.6 ± 4.1 (39)	6.3 ± 3.0 (49)
CANTAB SSP length (forward)	Baseline	3.5 ± 1.0 (27)	3.2 ± 1.1 (33)
	6 Months	3.6 ± 0.9 (26)	3.3 ± 1.3 (33)
CANTAB SSP length (reverse)	Baseline	2.5 ± 1.7 (27)	2.2 ± 1.4 (33)
	6 Months	2.8 ± 1.2 (26)	2.2 ± 1.4 (33)
CELF-P-2 (expressive)	Baseline	9.0 ± 5.6 (49)	12.9 ± 5.7 (41)
	6 Months	10.5 ± 5.5 (49)	12.0 ± 6.5 (39)
CELF-P-2 (receptive)	Baseline	14.5 ± 4.8 (49)	16.3 ± 4.6 (41)
	6 Months	14.8 ± 4.6 (49)	15.7 ± 6.0 (39)
BRIEF-P (composite)	Baseline	104 ± 16.4 (34)	92.1 ± 20.9 (27)
	6 Months	101 ± 16.0 (34)	91.2 ± 20.8 (26)
OMQ-PF	Baseline	94.4 ± 13.0 (49)	99.1 ± 13.0 (34)
	6 Months	93.9 ± 17.0 (48)	100 ± 14.3 (37)

IQ was not related to GEC scores ($p = 0.931$, **Table 5**). To further explore this lack of influence of IQ, correlations between IQ scores and the Working Memory domain, the Plan/Organize and the Inhibit domains were conducted and did not show any relation, in either age group. No significant correlations were found between BRIEF GEC scores and either forward or reverse span lengths from the CANTAB SSP tasks. Nevertheless, BRIEF-P Working Memory scores correlated with reverse SSP length ($R = -0.27$, $p = 0.036$, moderate effect).

Language Assessments

RBANS (Picture Naming and Semantic Fluency)

The baseline distribution of scores for both subtests followed normal distribution for both age groups, and a small number of participants performed at the floor of the tests (**Table 3**). No age differences were detected. Whereas, no effect of time was noticed in the Picture Naming task, time had a significant effect on Semantic Fluency results with lower scores at 6 months than at baseline (-1.17, $p < 0.001$, **Table 5**). Both Picture Naming and Semantic Fluency scores were significantly related to IQ ($p = 0.005$ and $p = 0.006$, respectively).

CELF-P-2 (Linguistic Functioning)

The baseline distribution of total scores in the CELF-P-2 was normal for the adolescents but was skewed toward the higher values for adults. This is likely due to a significant number of adult participants ($n = 12$) reaching the maximum score (or close to) of 20 for the receptive domain (but not for the expressive). Of note, female participants had a statistically higher average total scores (+3.95, $p = 0.037$) and expressive scores (+2.51, $p = 0.016$) than males. No gender differences were observed in receptive scores, likely due to the ceiling effect in this domain. Time did not affect any of the CELF-P-2 subscores. The total CELF-P-2 scores were significantly related to IQ scores ($p = 0.001$), driven by both the expressive and the receptive domains ($p = 0.003$; $p = 0.024$, respectively). To better understand the minimum level of language skills required to perform key cognitive tasks, we tested for correlations between receptive and expressive components of the CELF-P-2 and the RBANS List Learning and Semantic Fluency scores. In both age groups, CELF-P-2 expressive scores highly correlated with RBANS Semantic Fluency scores ($p < 0.001$) and with RBANS List Learning scores (**Figure 1**).

Test-retest Reliability

A summary of ICCs for all scales is shown in **Table 3**. Reliability ranged from fair (ICC 0.40–0.59) to very good (ICC > 0.75). Most of the scales depicted good reliability (ICC = 0.63: CELF-P-2, RBANS Semantic Fluency, List Learning and Story Memory subtests, BRIEF-P and OMQ-PF). The highest ICC scores were found for the BRIEF-P and OMQ-PF, which are both parent-reported scales.

DISCUSSION

This study assessed a variety of neurocognitive tests and functioning scales over a 6-month period to determine

TABLE 5 | Estimates of differences for each assessment and influence of Time, Age, and IQ (*p*-values as from MMRM analysis).

	BRIEF-P (composite)	CELF-P-2 (expressive)	CELF-P-2 (receptive)	CANTAB SSP length FW	CANTAB SSP length REV	OMQ-PF	RBANS Picture naming	RBANS Semantic fluency	RBANS List learning	RBANS Story memory
ESTIMATES										
6 Months –Baseline	−1.79	0.39	−0.08	0.16	0.25	0.21	0.16	−1.17	1.20	−0.81
Adults –Adolescents	−13.42	2.15	1.16	0.43	0.77	6.09	0.30	0.87	3.17	1.03
Females—Males	−0.63	2.51	1.30	−0.22	−0.25	2.80	0.71	1.82	2.09	0.74
TESTS OF EFFECTS										
Time	0.291	0.373	0.844	0.147	0.155	0.824	0.513	**<0.001**	0.051	**0.025**
Age	**0.011**	0.056	0.261	0.095	**0.019**	0.075	0.519	0.297	**0.035**	0.250
Time × Age	0.684	**0.014**	0.387	0.814	0.435	0.387	0.752	0.417	0.078	**0.030**
IQ	0.931	**0.003**	**0.024**	**<0.001**	**0.001**	0.331	**0.005**	**0.006**	**<0.001**	**0.001**

Bold values correspond to $p < 0.05$.

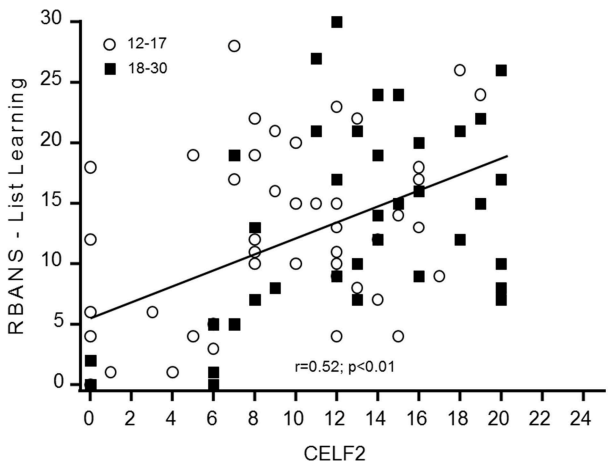

FIGURE 1 | RBANS List Learning scores and CELF-P-2 Word Classes expressive scores.

appropriate outcome measures for potential use in interventional pharmacological and non-pharmacological treatment studies in adolescents and young adults with DS. To date, this is the largest data set reporting evaluation of these assessments.

The Leiter-R IQ scale is a non-verbal assessment that is not influenced by linguistic production which is particularly impaired in individuals with DS. Moreover, in an international clinical trial context, form equivalence after language translation is a major barrier to the implementation of IQ scales. The Leiter-R is not influenced by this issue. Our results show that the Leiter-R may not be the most suitable means of capturing the lower end of the IQ range in DS as 22% of adolescents and 61% of adults scored at the floor of the test (36); however, this test has shown better results than those obtained in a previous clinical trial with the abbreviated Stanford-Binet Intelligence Scales Fifth Edition (ClinicalTrials.gov Identifier: NCT01436955). Based on these observations, the Leiter-3 (Roid and Miller, 1997) was administered in a study with 180 adults and adolescents with DS (Clinical.Trials.gov Identifier NCT01920633). These results are more promising in terms of data distribution and percentage of participants at the floor of 30 (approximately 1%). This suggests that the Leiter-3 is probably more appropriate to measure the full IQ range in this population (**Figure 2**). In studies in children with DS, it is not uncommon for standardized IQ scores to decrease across childhood (Carr, 1995). In our study of older individuals with DS (12–30 years), using the Leiter-R we found stability in IQ scores similar to the recent findings by Carr, showing no change in IQ from 21 to 45 years in a longitudinally collected sample(Carr, 2012). However, with the greater number of adults at the floor of 36, any age-related differences may have been masked by floor effects.

The RBANS was developed for the dual purposes of identifying and characterizing abnormal cognitive decline in older adults and as a neuropsychological screening battery for younger patients (Randolph et al., 1998). With average List Learning scores of 14 for adults with DS where the average score in typically developing peers is approximately 30, and even greater discrepancies in the Story Memory subtest, this demonstrates that these tasks are very difficult for individuals with DS. Some improvements in performance were observed over the 6-month study period and, in particular, adolescents showed improvement in the List Learning task. These observations may be linked to the natural neurodevelopment of the capacities of adolescents and/or the fact that more adolescents with DS are attending school and involved in alternative therapies such as speech therapies and educational resources. The Story Memory scores, however, did not show a similar improvement in adolescents which may be due to a greater floor effect.

Observed memory is not a direct measure of the participant's memory capacities, but a functional measure that can be affected by many facets of mnemonic ability in daily life. Overall, the OMQ-PF showed good reliability and suitability for use in clinical trials of individuals with DS. Previous results by Gonzales et al. have indicated that the OMQ-PF may be more

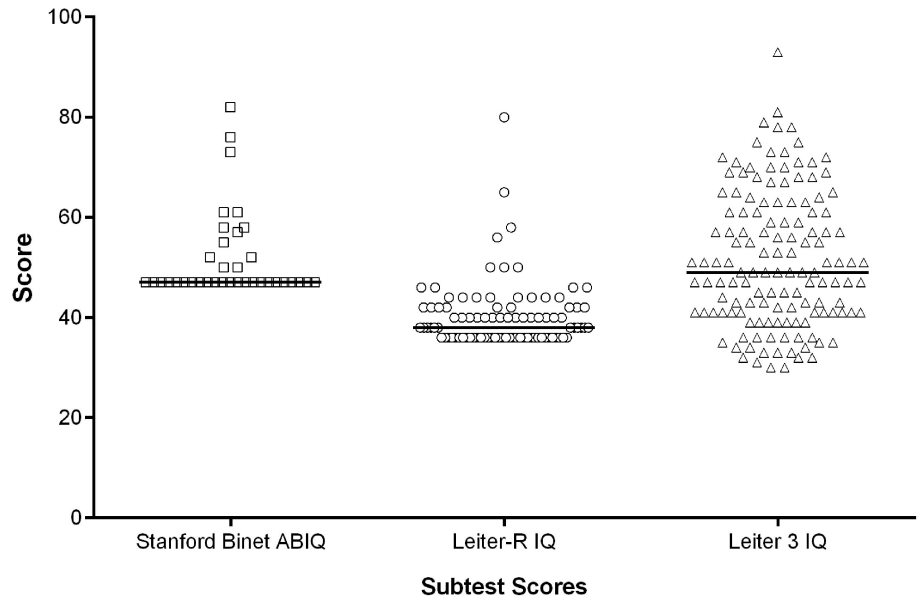

FIGURE 2 | IQ scores: distributions using three different IQ scales. The horizontal bar represents the median score.

closely related to new learning ability rather than retention or recall of information (Gonzalez et al., 2008), and other studies suggest that everyday abilities cannot necessarily be inferred from direct neuropsychological tasks (Chaytor and Schmitter-Edgecombe, 2003). Our results suggest that observed memory scores relate to specific memory functions, as illustrated by the correlation noted between the RBANS List Learning immediate and OMQ-PF scores.

Forward and backward SSP subtests were administered to assess working memory. Here we recapitulate the known working memory deficit in DS (Lanfranchi et al., 2012), with low scores in both forward and reverse tasks and a significant number of participants scoring 0, or "floor" effects, in the reverse task. Both subtests were statistically related to IQ scores, however, this relationship is likely driven by the floor effects in both IQ and spatial span tests, and thus less meaningful. Neither forward nor reverse SSP length correlated with BRIEF-P GEC scores. Overall, these findings together with low ICC values indicate that SSP would be too difficult and discouraging for individuals with DS and have limited usefulness as an outcome measure in interventional clinical trials.

The BRIEF-P was implemented as an indirect measure of executive function, including working memory function. Here again executive function deficits were clear, confirming the neurocognitive DS profile. An obvious difference was evident between the adolescent and adult groups in GEC scores, with adults performing significantly better than adolescents. Adult performance reached maximum scores, suggesting that the preschool version of the BRIEF is probably less appropriate for the adults than the adolescents with DS. The BRIEF-school age version (5–18 years) could have been used instead. This version of the BRIEF was indeed used as a behavioral assessment to establish concurrent validity for the ACTB (Edgin et al., 2010a). The perceived global executive function was not influenced by IQ across ages. We therefore looked at IQ correlations in adolescents and adults separately in BRIEF-P subdomains and interestingly noted that neither, the Working Memory, Plan/Organize or Inhibit subtests correlated with IQ. However, a focused analysis of Working Memory aspects, considered to be a major contributor to executive function weaknesses in DS, revealed that the Working Memory domain of the BRIEF-P correlated with reverse SSP, a direct Working Memory executive function measure. These findings suggest that the BRIEF-P captures executive functions engaged in the reverse SSP processing, but overall distinct functions than those captured by the Leiter.

Language difficulties are one of the most prominent barriers to independence and socialization and part of the neurocognitive profile in DS. Here we assessed elements of linguistic functioning. The CELF-P-2 Word Classes test showed a potential "ceiling" effect, reducing its use to assess changes in language abilities in a trial; nevertheless, the link between CELF-P-2 expressive scores and RBANS List Learning performances suggests this test could be of relevant use as a screening tool in future studies to ensure enrolment of participants with the minimal level of expressive language ability required to perform key cognitive tasks. In our study, the verbal communication level was on average better in females as compared to males, particularly in the expressive domain, as assessed by the CELF-P-2 Word Classes and RBANS Semantic Fluency, confirming the previously described communication profile in DS (Määttä et al., 2006).

Language proficiency was also tested with the Picture Naming and Semantic Fluency tasks from the RBANS. Overall, the test-retest scores from these two tests were considered fair, illustrating a potential lack of suitability for clinical trials in individuals with DS. However, to avoid potential practice effects, four different

TABLE 6 | Summary of key learnings.

Domain	Test name	Suitable for clinical trials with people with DS		Summary of main findings
		12–17 years	18–30 years	
IQ measurement	Leiter-R	No	No	- Floor effect observed at 36
	Leiter-3	Yes	Yes	- No floor effect, good distribution
Memory	RBANS—Short term memory			- Differences between the forms
	List learning	Yes	Yes	- Good test-retest reliability, no floor effect, sensitive to age and IQ
	Story memory	No	Yes	- Floor effects and unstable over time in adolescents.
	OMQ-PF	Yes	Yes	- Good stability over time and good test-retest reliability
Executive function	CANTAB SSP			
	Forward	Yes	Yes	- No floor effect, good test-retest reliability, sensitive to IQ
	Reverse	No	No	- Floor effects in both age groups, low reliability
	BRIEF-P	Yes	No	- Reliable, stable and sensitive to age and detects impairment in the working memory domain - Ceiling effect in adults
Language	CELF-P-2 Word classes	Yes	Yes	- Stable, reliable and sensitive to age and IQ - Ceiling effect in the receptive domain in adults (recommend to use CELF-4)
	RBANS			
	Semantic fluency	Yes	Yes	- No floor effect, sensitive to spoken language and IQ but not age
	Picture naming	No	No	- Low test-retest reliability

RBANS forms have been developed to be used on several occasions in clinical trials. A weakness in our study is that the same RBANS form was used at the baseline visit but two different forms were used at the Week 24 visit depending on the study schedule. This might explained the low ICC scores that we observed or the time effect observed in the Semantic Fluency task.

Finally, we observed that direct measurements of immediate memory, executive function and linguistic functioning as described here, were all influenced by the IQ level of the participants. On the other hand, indirect measures of executive function and memory as reported by the parents or the caregivers (BRIEF-P and OMQ-PF) were not sensitive to the IQ level.

Table 6 summarizes the main findings for each scale evaluated in this study and our conclusions on their suitability for clinical trials with adults and adolescents with Down syndrome. These conclusions contributed to the selection of suitable outcome measures for the ongoing 26-week Phase 2 study (Clinicaltrials.gov identifier: NCT02024789) evaluating the efficacy, safety and tolerability of Basmisanil in individuals (12–30 years) with DS. RBANS List Learning was chosen as the primary endpoint for evaluating hippocampal tasks associated with a global functioning evaluation, whereas the Leiter-3 was selected as the IQ measure. These results can be relevant to other trials assessing cognitive function in the DS population, but also in other conditions. Given the breath of these measures we have validated scales that could be used across trials, including memory interventions (RBANS, OMQ-PF) as well as in attention deficits (BRIEF-P, CANTAB spatial span).

CONCLUSION

To our knowledge, the results reported here are the first from a multinational study assessing cognitive function in a substantial number of adolescents and adults with DS over a 6-month period, allowing both robust suitability and reliability analyses. Multiple assessments that evaluate overlapping cognitive functions were conducted, which allowed for a robust characterization of these scales and their interrelationships. Finally, these findings provide information on the natural neurocognitive changes in adolescents and adults with DS over a 6-month period, which will contribute to a better understanding of the true impact of intervention in future efficacy trials.

Overall, the current study has important implications for measuring cognitive changes in response to pharmacological treatment. Such non-pharmacological, longitudinal studies are key in the development of medicine for neurodevelopmental disorders such as DS where the choice of appropriate tools is critical to be able to detect beneficial drug effects.

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frontiers in Behavioral Neuroscience

ORIGINAL RESEARCH
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The down syndrome biomarker initiative (DSBI) pilot: proof of concept for deep phenotyping of Alzheimer's disease biomarkers in down syndrome

Michael S. Rafii[1], Hannah Wishnek[1], James B. Brewer[1], Michael C. Donohue[2], Seth Ness[3], William C. Mobley[1], Paul S. Aisen[2] and Robert A. Rissman[1]*

[1] Alzheimer's Disease Cooperative Study, Department of Neurosciences, UC San Diego School of Medicine, University of California, San Diego, La Jolla, CA, USA, [2] Department of Neurology, University of Southern California, Los Angeles, CA, USA, [3] Janssen Research and Development LLC, Titusville, NJ, USA

To gain further knowledge on the preclinical phase of Alzheimer's disease (AD), we sought to characterize cognitive performance, neuroimaging and plasma-based AD biomarkers in a cohort of non-demented adults with down syndrome (DS). The goal of the down syndrome biomarker Initiative (DSBI) pilot is to test feasibility of this approach for future multicenter studies. We enrolled 12 non-demented participants with DS between the ages of 30–60 years old. Participants underwent extensive cognitive testing, volumetric MRI, amyloid positron emission tomography (PET; 18F-florbetapir), fluorodeoxyglucose (FDG) PET (18F-fluorodeoxyglucose) and retinal amyloid imaging. In addition, plasma beta-amyloid (Aβ) species were measured and Apolipoprotein E (ApoE) genotyping was performed. Results from our multimodal analysis suggest greater hippocampal atrophy with amyloid load. Additionally, we identified an inverse relationship between amyloid load and regional glucose metabolism. Cognitive and functional measures did not correlate with amyloid load in DS but did correlate with regional FDG PET measures. Biomarkers of AD can be readily studied in adults with DS as in other preclinical AD populations. Importantly, all subjects in this feasibility study were able to complete all test procedures. The data indicate that a large, multicenter longitudinal study is feasible to better understand the trajectories of AD biomarkers in this enriched population. This trial is registered with ClinicalTrials.gov, number NCT02141971.

Keywords: down syndrome, Alzheimer's disease, biomarkers, amyloid, MRI, PET, plasma, retinal

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****Correspondence:***
Michael S. Rafii, Alzheimer's Disease Cooperative Study, Department of Neurosciences, UC San Diego School of Medicine, University of California, San Diego, 9500 Gilman Drive, MC 0949, La Jolla, CA 92094-0949, USA
mrafii@ucsd.edu

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Introduction

The preclinical/asymptomatic stage of Alzheimer's disease (AD) has become a target for therapeutic intervention, requiring enriched populations to be more intensively studied. Individuals with Down Syndrome (DS) comprise the largest group with genetically determined AD, with a worldwide population of about six million people. In March 2013, the Alzheimer's Disease Cooperative Study (ADCS) launched a pilot study named the Down Syndrome Biomarker Initiative (DSBI; Ness et al., 2012). With the DSBI pilot, the ADCS' goal was to initiate a longitudinal biomarker

study similar to the Alzheimer's Disease Neuroimaging Initiative (ADNI) in individuals with DS, who represent a population highly enriched for developing AD. The ultimate aim of this work is to aid the development of preventive therapies for the dementia associated with both DS and AD, based on the apparent common pathogenic role of beta amyloid (Aβ) in the two conditions.

The tight link between genetic determinants of AD and the overproduction of Aβ provides compelling support for the amyloid cascade hypothesis and has been the focal point in the development of disease-modifying drugs for AD (for review, Sperling et al., 2011). We hypothesize that disease-modifying treatments for AD and DS should begin prior to the onset of cognitive symptoms to prevent extensive neurodegeneration and thus necessitate a clear understanding of biomarker changes throughout the course of the disease.

The study of DS provides a unique opportunity to characterize the preclinical changes associated with predisposition to AD. DS, or trisomy 21, affects 400,000 people in the U.S. with an incidence of 1/691 live births (Parker et al., 2010) and is caused by meiotic non-disjunction, leading to an extra copy of chromosome 21, on which the APP gene resides.

Recent data suggest that AD biomarker changes in DS are similar to those observed in familial and sporadic AD. For example, studies demonstrate a six-fold increase in plasma Aβ in individuals with DS as compared to age-matched non-DS individuals (Schupf et al., 2001, 2007, 2010) and Aβ positron emission tomography (PET) imaging data in DS are consistent with AD patients (Sabbagh et al., 2011, 2015; Handen et al., 2012). Furthermore, as seen in familial and sporadic AD, presence of the Apolipoprotein E (ApoE) ε4 allele is generally associated with greater accumulation of Aβ plaques in the brains of adults with DS (Hyman et al., 1995; Lemere et al., 1996). Presence of ApoE ε4 allele is also associated with an earlier age of onset of dementia (Schupf et al., 1996; Deb et al., 2000; Coppus et al., 2008; Prasher et al., 2008).

Postmortem studies indicate that adults with DS have a similar, prominent pattern of cerebral atrophy involving the medial temporal lobe structures, as seen in the early stages of AD (Hof et al., 1995; Teipel et al., 2004; Mullins et al., 2013). Volumetric magnetic resonance imaging (MRI) studies of age-related brain changes in DS demonstrate the same pattern of hippocampal-specific atrophy observed in AD. Furthermore, the hippocampal atrophy in DS correlates with changes in memory measures (Krasuski et al., 2002; Beacher et al., 2010). Hypometabolism on regional fluorodeoxyglucose (FDG) PET also correlates with onset of dementia in older adults with DS (Schapiro et al., 1992a,b; Pietrini et al., 1997).

In this study, we collected structural MRI, Aβ PET, FDG PET, retinal Aβ, plasma Aβ species, and cognitive performance measurements in a cohort of 12 non-demented adults with DS aged 30–60. Our goal was to establish feasibility of conducting a biomarker-intensive study in adults with DS.

Materials and Methods

Study Design and Participants

The DSBI pilot enrolled 12 non-demented subjects for a 3-year longitudinal study of AD biomarkers (see **Table 1** for Schedule of Events). The present analysis is restricted to the baseline data. Four non-demented subjects were in each age range: 30–40, 40–50 and 50–60. Inclusion criteria limited enrollment to individuals having a chromosome karyotype of DS due to Trisomy 21. Subjects were required to have a caregiver, absence of other neurological and psychiatric disorders, and be capable of and willing to perform study procedures. Having a clinical diagnosis of dementia was considered exclusionary as was presence of 6 months of progressive cognitive or functional decline as per ICD-10 criteria (Sheehan et al., 2015). Exclusion of a diagnosis of dementia was also based on absence of evidence of recent deterioration in cognitive function found not secondary to medical illness (e.g., hypothyroidism, sleep apnea) in conjunction with absence of a significant decline in function over a period of 6 months or more. The diagnosing neurologist was experienced with dementia in DS and incorporated diagnostic recommendations from the National Task Group on Intellectual Disabilities and Dementia Practices (Moran et al., 2013). All participants or their legal representatives provided written informed consent before partaking in the study in accordance with the regulations and approval of the ethics committee

TABLE 1 | Schedule of events for DSBI pilot.

Visit	Screen/ BL	YR1	YR2	YR3 (Comp)
Month	0	12	24	36
Study Procedures				
Screening/administrative				
Informed consent [/assent]	x			
Inclusion/exclusion criteria	x			
Medical history and demographics	x			
Safety assessments				
Physical examination	x	x	x	x
Vital signs	x	x	x	x
Neurocog assessments				
Scales, questionnaires, etc.	x	x	x	x
Clinical laboratory assessments				
Hematology, Chemistry	x	x	x	x
Urinalysis	x	x	x	x
Pharmacogenomics (DNA)				
ApoE	x			
Biomarkers (eg, plasma, serum sample collection)				
Plasma, serum collection	x	x	x	x
Imaging				
Tau PET			x	x
Amyvid PET	x			x
FDG PET	x			x
vMRI	x	x	x	x
Retinal amyloid imaging	x	x	x	x
Ongoing subject review				
Concomitant therapy	x	x	x	x
Adverse events	x	x	x	x

at the University of California, San Diego, La Jolla, CA, USA.

Procedures

Between March 2013 and January 2014, we collected data from participants including plasma samples, neuropsychological evaluations, neurological examination, ApoE genotyping, volumetric MRI, amyloid PET, FDG PET, retinal Aβ imaging, and clinical assessment. Subjects came for five visits over a 5-week period for assessments to be made. Events occured in the following order: visit 1: neuropsychological and clinical assessment, neurological examination; visit 2: Amyloid PET; visit 3: MRI and blood draw; visit 4: FDG PET, visit 5: retinal Aβ imaging.

Cognitive, Behavioral, and Functional Assessments

Cambridge Neuropsychological Test Automated Battery (CANTAB). The CANTAB was used to assess cognition. The CANTAB is a computerized touch-screen assessment of neuropsychological function composed of a number of tests (Luciana, 2003; Smith et al., 2013). The tests selected from this battery for this study were as follows: motor control (MOT): the subject is asked upon appearance of a crossmark on the screen, to touch it as quickly and accurately as possible using the index finger of their dominant hand. This is essentially a practice routine to become skilled with regards to touchscreen use. The outcome parameter is median reaction time (RT): the subject is asked to hold the index finger on the holding button on the button box and keep it pressed until a circle on the screen lights up and then touch that circle with the index finger as quickly and accurately as possible. In the Simple condition, there is only one possible circle that will light up (Simple RT). In the five-choice condition, any of five circles can light up (five-Choice RT). Paired associated learning (PAL): the subject is shown 2–8 (max) distinct visual patterns, each at one of eight positions inside of an octagon on the screen. The task is to memorize which pattern occurred where. After the memorization stage, each pattern is shown in the center of the screen and the subject has then to touch one of eight possible positions where the pattern first occurred.

Repeatable Battery for the Assessment of Neuropsychological Status (RBANS) was developed for the dual purposes of identifying and characterizing abnormal cognitive decline in the older adult and as a neuropsychological screening battery for younger patients (Randolph et al., 1998). It is a brief, individually administered test that can be used to measure cognitive decline or improvement. The full battery is composed of 12 subtests assessing the: immediate memory, visuospatial abilities, language, attention and delayed memory. In this study, seven subtests of the RBANS were used to assess immediate and delayed memory, as well as the language capacities (subtests: list learning, story memory, list recognition, list recall, picture naming, semantic fluency, digit span).

Vineland-II Adaptive Behavior Scale (VABS-II) parent/caregiver interview form. The VABS-II measures personal and social skills such as communication, daily leaving skills, and socialization and will provide a composite score reflecting an individual's overall function. In addition, the optional maladaptive behavior index could be used. The survey interview form was administered to parents or caregivers using a semi-structured interview format (Sparrow and Havis, 2005).

Observer Memory Questionnaire-Parent Form (OMQ-PF). The OMQ-PF is a 27-item questionnaire designed to ascertain parents' perceptions of the subject's memory function. This questionnaire is comprised of items inquiring about memory function in everyday scenarios (Gonzalez et al., 2008).

Anxiety Depression and Mood Scale (ADAMS). The ADAMS is a well validated, 28 item behavior-based informant instrument designed to be used specifically with individuals with developmental disabilities to assess anxiety, depression and mood disorders (Esbensen et al., 2003). Points given for each behavior the caregiver endorses. Subscales (5) include: Manic/Hyperactive, Depressed Mood, Social Avoidance, General Anxiety, Compulsive Behavior. The ADAMS possesses a satisfactorily high alpha, with a mean alpha of 0.80 in each of the 28 items. The mean item test-retest correlation is 0.789.

Cambridge Examination for Mental Disorders of Older People with Down's Syndrome and Others with Intellectual Disabilities (CAMDEX-DS). Cognitive status was measured using the Cambridge Cognitive Examination (CAMCOG), the cognitive section of CAMDEX, a composite index of episodic memory, orientation, language, attention, praxis and executive function previously validated for use in DS (Hon et al., 1999). The CAMCOG is appropriate for assessing cognitive function in people with intellectual disability, unlike more standard tests of cognitive function such as the Wechsler Adult Intelligence Scales. The CAMCOG incorporates, and is highly correlated with, the Mini Mental State Examination (MMSE; Blessed et al., 1991).

Dalton Dyspraxia scale for Adults with DS: evaluates simple sequences of voluntary movements expected to deteriorate with the onset and progression of dementia in AD among persons at all levels of premorbid intellectual disability. Participants are given points for each task they are able to perform (Dalton, 1992).

The Goodenough–Harris Draw-A-Person Test: brief paper and pencil mental age test. This assessment system analyses 14 different aspects of a drawing done by the subject (such as specific body parts and clothing) for various criteria, including presence or absence, detail, and proportion. In all, there are 64 scoring items. A standard score is recorded for the drawing, and a mental age is assigned based on this score (Goodenough and Harris, 1950).

Biofluid Collection

Blood, (separated into plasma and serum), was collected to accommodate the assay of the broadest range of the best antecedent biomarkers/analytes. Blood samples were drawn in

two lavender-capped EDTA tubes and one red-capped BD tube. One lavender-capped tube was centrifuged at 3000 rpm for 10 min to separate plasma for storage. Ten milliliter of the plasma sample was aliquoted into barcoded polypropylene vial and frozen at −80. The second blood tube was used for serum extraction, which will be processed by allowing the samples to clot at room temperature, spun as above for plasma preparation, aliquoted and stored in barcoded polypropylene tubes at −80. The third blood tube was used for DNA isolation using Qiamp DNA blood maxi kit (Qiagen). All biosamples were processed and stored at the ADCS Biomarker Core using standard operating procedure.

Plasma Aβ Analysis and Internal Standard

Banked plasma was assayed, quantified, and quality controlled by the ADCS Biomarker Core using the MesoScale Validated Aβ triplex (Aβ 38, 40, 42) according to the manufacturer instructions. Each assay plate also included an internal standard which provided a means for adjusting plate-to-plate variation and assessing freezer storage effects, as previously described (Donohue et al., 2014). To mitigate plate-to-plate variability, plates were purchased in bulk and run consecutively.

Real Time PCR for Apolipoprotein E (ApoE) Genotyping

Genotyping for ApoE alleles was performed using real time PCR Restriction Fragment Length Polymorphism analysis by the ADCS Biomarker Core according to standard operating procedures. ApoE genotyping was performed using Applied Biosystems TaqMan SNP Genotyping Assay (C_3084793_20 and C_904973_10 corresponding to ApoE SNPs rs429358 and rs7412, respectively). The assay was run on a Bio-Rad CFX96 Touch Real Time PCR Detection System, using a cycling program of 98 C for 2 min. and 39 cycles of 98 C for 15 s and 62 C for 45 s five positive controls for each genotype and one negative control were included in each plate to ensure accurate determination.

Neuroimaging

Volumetric MRI

The MRI protocol included series to assess for structural pathology (T2-weighted fluid attenuated inversion recovery, T2*-weighted gradient recalled echo, and diffusion weighted imaging) along with a series modeled on the non-accelerated T1-weighted sequence from ADNI for volumetric processing (3D inversion recovery prepared spoiled gradient recalled imaging; inversion time 500, flip angle 10, 1.25 mm × 1.25 mm in-plane resolution, 156 sagittal slices with 1.2 mm spacing). Scanning was performed on a 1.5 Tesla GE Signa HDxt scanner, and radiologist overread was performed on all scans to identify any clinically significant incidental findings. NeuroQuant image preprocessing and automated segmentation was used to measure brain structure volumes (Brewer et al., 2009; Kovacevic et al., 2009; Heister et al., 2011). Briefly, this includes corrections for gradient non-linearities (Jovicich et al., 2006) and intensity non-uniformity (Sled et al., 1998) and application of probabilistic-atlas-based segmentation to automate measurement of multiple brain regions (Fischl et al., 2002). The procedure is cleared by the U.S. Food and Drug Administration and the European Medicines Agency for use in automating the identifying, labeling, and quantifying the volume of segmental brain structures identified on MR images (21 CFR 892.2050). To minimize multiple comparisons, for analysis, a single measure of medial temporal atrophy that comprises hippocampal volume loss and temporal horn ex-vacuo dilatation, "Hippocampal occupancy (HOC)," was calculated as described previously (Heister et al., 2011). This measure is simply H/(H + T), where H is hippocampal volume and T is temporal horn volume.

FDG PET

FDG PET procedures were based on those used in ADNI.[1] Subjects were asked to fast for at least 6 h prior to the scanning session. Subjects' blood glucose was checked prior to scanning and was required to be <180 mg/dL. After the injection of 5 mCi of 18F-FDG, subjects were kept in a quiet, dimly lit room with eyes and ears unoccluded for 30 min, after which they were placed in the Siemens EXACT HR+ 961 PET tomograph (CTI, Knoxville, TN, USA), which yielded 63 transverse sections spaced 2.43 mm apart with a 15.5 cm field of view (FOV) in 3D mode and 5 mm in-plane spatial resolution full width at half maximum (FWHM). Images were acquired at an angle parallel to the cantho-meatal plane and reconstructed using a ramp filter (cut-off frequency = 0.5 cycles/pixel) into 128 × 128 pixel images. Each subject was placed in a headholder during scanning to allow accurate positioning using a low-power neon laser. Data were acquired as 6 × 5 min frames, followed by a positron transmission scan. Frames were averaged and all images were coregistered to the individual's native space MRI. For signal normalization, the brainstem was used as a reference region.

Florbetapir F 18 PET

Subjects received IV injections of 10 mCi of Florbetapir F 18 and after 40 min of uptake, 10 min of emission data were collected by the Siemens EXACT HR+ 961 PET tomograph (CTI, Knoxville, TN, USA), which yielded 63 transverse sections spaced 2.43, 3.5 mm apart with a 15.5 cm FOV in 3D mode, with 4 mm in-plane spatial resolution (FWHM). Images were acquired at an angle parallel to the cantho-meatal plane and reconstructed using a Hann filter (cut-off frequency = 0.5 cycles/pixel) into 128 × 128 pixel images. Each subject was placed in a headholder during scanning to allow accurate positioning using a low-power neon laser. All PET scans were supervised. Statistical analysis was performed as for FDG, except, for florbetapir, the cerebellum was used as the reference region for signal normalization.

[1]http://www.adni-info.org/Scientists/doc/ADNI2_PET%20Tech_Manual-Version_4_2014Oct27_CLEAN.pdf

TABLE 2 | Participant characteristics in DSBI feasibility study.

	N (E4−)	N (E4+)	Total (*N*)
ApoE	6	6	12
Gender: F	6	4	10
Gender: M	1	1	2
Age	43.5 (9.8)	47.2 (7.4)	45.0 (9.8)
Educ. years:			
0	0	1	1
12	2	5	7
18	4	0	4

There were six subjects who were ApoE4 positive and six who were ApoE4 negative. Average age was 45 (S.D. 9.8).

Retinal Aβ

The NeuroVision Retina HD is a fundus camera that is substantially equivalent to the FDA approved cameras currently utilized in clinical practice. In this procedure, a filter set matched to the fluorescence characteristics of curcumin is utilized for retinal amyloid plaque imaging *in vivo*. Quantitative analysis of Aβ plaque number, area (μm^2) and distribution are performed from retinal images. For the acquisition, the same exposure settings and the same gain values are used for all images. The emission signals of Aβ plaques stained with curcumin are compared to the background signals in the retinal tissue, to determine signal-to-background ratio.

At the visit, subjects had auto-fluorescence imaging and curcumin fluorescence imaging of the right retina. Patients were asked to take a standard over the counter oral vitamin E supplement for each retinal amyloid imaging visit, beginning at Day 1 and continuing through day 3 of imaging. Patients were dosed with curcumin twice daily for 2 days; At Day 1, patients commenced taking oral curcumin. On Day 2, subjects had another day of ingesting curcumin. On Day 3, subjects had auto-fluorescence imaging, and curcumin fluorescence imaging. NeuroVision calculated the retinal amyloid index in a blinded fashion for each subject.

Statistical Analysis

For cognitive, imaging analyses and fluid biomarker assessment, ApoE4 carriers and non-carriers were compared in terms of their age, educational level, clinical ratings, and neuropsychological test scores using Wilcoxon and Pearson's Chi-square tests. We also estimated Spearman rank correlations for each selected pairs of continuous measures. These correlation analyses were group by variable type: (1) cognitive vs. imaging; (2) cognitive vs. retinal and plasma; and (3) MRI vs. PET. We controlled the false discovery rate within each of these groups (Benjamini and Hochberg, 1995). This pilot study is not well powered. All analyses should be considered exploratory and any findings need to be confirmed with larger sample sizes. The sample size of $n = 12$ provides approximately 80% power to detect only correlations larger than $\rho = 0.84$ with two-sided FDR $\alpha = 5\%$ and assuming 90% of null hypotheses are true. Similarly, $n = 6$ subjects per ApoEε4 group provides approximately 80% power to detect only large standardized group differences of $\delta = 2.66$.

Results

All 12 participants completed all required testing. **Tables 2** and **3** show demographic characteristics for the 12 non-demented participants included in this study, grouped by APoE4. Of the 12 subjects, 50% ($n = 6$) were ApoE4 carriers. All of the ApoEε4 non-carriers were female, while four of the ApoEε4 carriers were female. These two groups did not differ significantly in demographics, clinical ratings, or neuropsychological test scores. All subjects were

TABLE 3 | Cognitive and functional performance summaries by ApoE4 geneotype.

	N	E4−	E4+	Combined	*P*-value
Direct testing					
CAMCOG-DS	12	58.2 (23.4)	56.5 (20.3)	57.3 (20.9)	0.81
Goodenough-DAP	12	14.83 (9.47)	17.33 (2.73)	16.08 (6.78)	0.81
RBANS Composite	12	259.3 (30.1)	249.7 (47.1)	254.5 (38.0)	0.47
RBANS Digit span	12	4.67 (2.07)	3.33 (2.66)	4.00 (2.37)	0.27
RBANS List recall	12	14.67 (3.98)	11.83 (3.54)	13.25 (3.89)	0.18
RBANS Memory	12	50.00 (6.66)	49.50 (13.17)	49.75 (9.96)	0.68
RBANS Language	12	70.8 (19.9)	60.7 (16.3)	65.8 (18.2)	0.22
Delayed Memory	12	43.00 (3.79)	43.17 (7.76)	43.08 (5.82)	0.44
CANTAB-Total	12	87.5 (33.2)	109.0 (33.6)	98.2 (33.8)	0.29
Dalton Dyspraxia	12	205.7 (41.3)	177.7 (49.9)	191.7 (46.1)	0.31
Informant-based					
ADAMS	12	17.8 (17.4)	17.8 (23.3)	17.8 (19.6)	0.81
Vineland-2	12	124.217 (32.9)	96.767 (24.5)	110.4 (31.2)	0.24
OMQ-PF	12	91.5 (27.1)	89.8 (27.3)	90.7 (26.0)	0.69

Higher score on all cognitive tests indicates better performance. The only exception is higher score on ADAMS, which indicates increased symptoms of anxiety, depression, agitation. ADAMS, Anxiety Depression and Mood Scale; CAMCOG-DS, Cambridge Examination for Mental Disorders of Older People with Down's Syndrome and Others with Intellectual Disabilities-Cognitive scale; Goodenough DAP, Goodenough-Harris Draw a Person test; RBANS, Repeatable Battery for the Assessment of Neuropsychological Status; (CANTAB), Cambridge Neuropsychological Test Automated Battery; OMQ-PF, Observer Memory Questionnaire (Parent Form).

TABLE 4 | Amyloid PET and FDG PET with hippocampal volume and retinal amyloid index.

Subject	Age	Mental age	ApoE 4	Amyloid PET clinical read	Grey matter Amyloid PET (SUVr)	FDG PET clinical read	Average Hippocampal volume (cm^3)	Average Hippocampal Occupancy (%)	Retinal amyloid index
DP06	37	9	E3-E3	Negative	0.938	Normal	3.52	73	1.63
DP01	32	7	E3-E3	Negative	0.97	Mildly hypo	3.12	78	2
DP07	34	7	E2-E4	Negative	0.988	Normal	3.37	75	2.47
DP08	39	5	E3-E3	Positive	1.054	Hypo	3.19	82	1.8
DP02	45	3	E2-E3	Positive	1.171	Hypo	2.99	45	2.2
DP12	45	6	E3-E4	Positive	1.176	Hypo	2.91	75	1.83
DP05	48	8	E3-E3	Positive	1.177	Hypo	3.47	77	1.68
DP11	47	7	E3-E4	Positive	1.245	Hypo	3.48	71	2.34
DP13	50	8	E3-E4	Positive	1.344	Hypo	3.14	60	1.58
DP04	55	6	E3-E4	Positive	1.385	Hypo	3.01	45	1.7
DP03	52	7	E3-E4	Positive	1.401	Hypo	3.25	51	2.2
DP09	60	7	E3-E3	Positive	1.457	Hypo	2.73	60	—

Areas with higher amyloid deposition have relative hypometabolism on FDG PET. The listing is sorted with respect to increasing grey matter amyloid PET signal.

amyloid positive, but to varying degrees. **Table 4** provides a line listing of some of the key neuroimaging variables for each study participant sorted by posterior cingulate gyrus (PCG) amyloid PET. **Figure 1** demonstrates how multimodal assessments are made across subjects in native space.

Correlation Analyses

Figure 2 shows the correlation between key neuroimaging, and age and cognition. Age was significantly associated with florbetapir (AV45) uptake in the gray matter ($r = 0.963$, $p < 0.001$) and thalamus ($r = 0.595$, $p < 0.041$), and HOC ($r = -0.662$, $p = 0.019$). Florbetapir uptake in the gray matter was also significantly correlated with OMQ PF ($r = -0.769$, $p = 0.005032$). FDG uptake in the thalamus was significantly correlated with CAMCOG, Digit Span, OMQ PF, RBANS, and Vineland (all $r > 0.6$ and $p < 0.01$). HOC was correlated with OMQ PF ($r = 0.587$, $p = 0.049$). Only the correlations between FDG Thalamus and OMQ-PF; FDG Thalamus and Vineland; and Florbetapir (AV45) Gray Matter and age were significant at the 0.005 level ($r = 0.776$, $r = 0.776$, and $r = 0.963$ respectively).

Correlation Between Cognition, and Plasma Aβ and Retinal Amyloid

Figure 3 shows the correlation between key neuroimaging, and age and cognition. We found no significant correlations between plasma or retinal amyloid measures

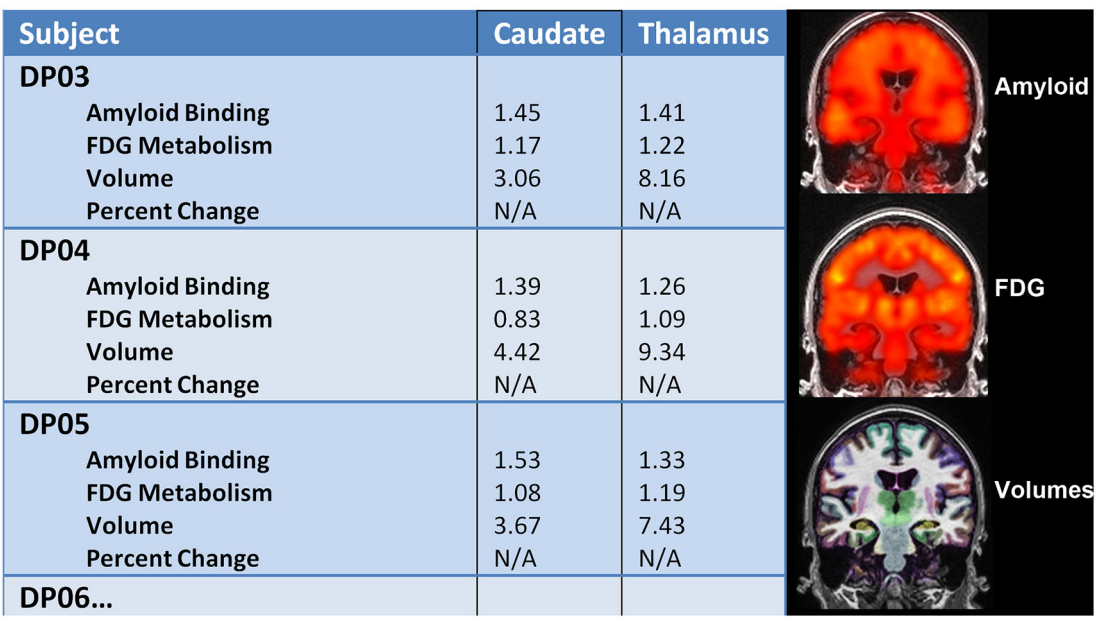

FIGURE 1 | Left: multimodal comparisons can be made in native space within individual subjects longitudinally. **Right:** Amyloid PET, FDG PET, and volumetric MRI were successfully performed in adults with down syndrome (DS) to capture important structure-function relationships.

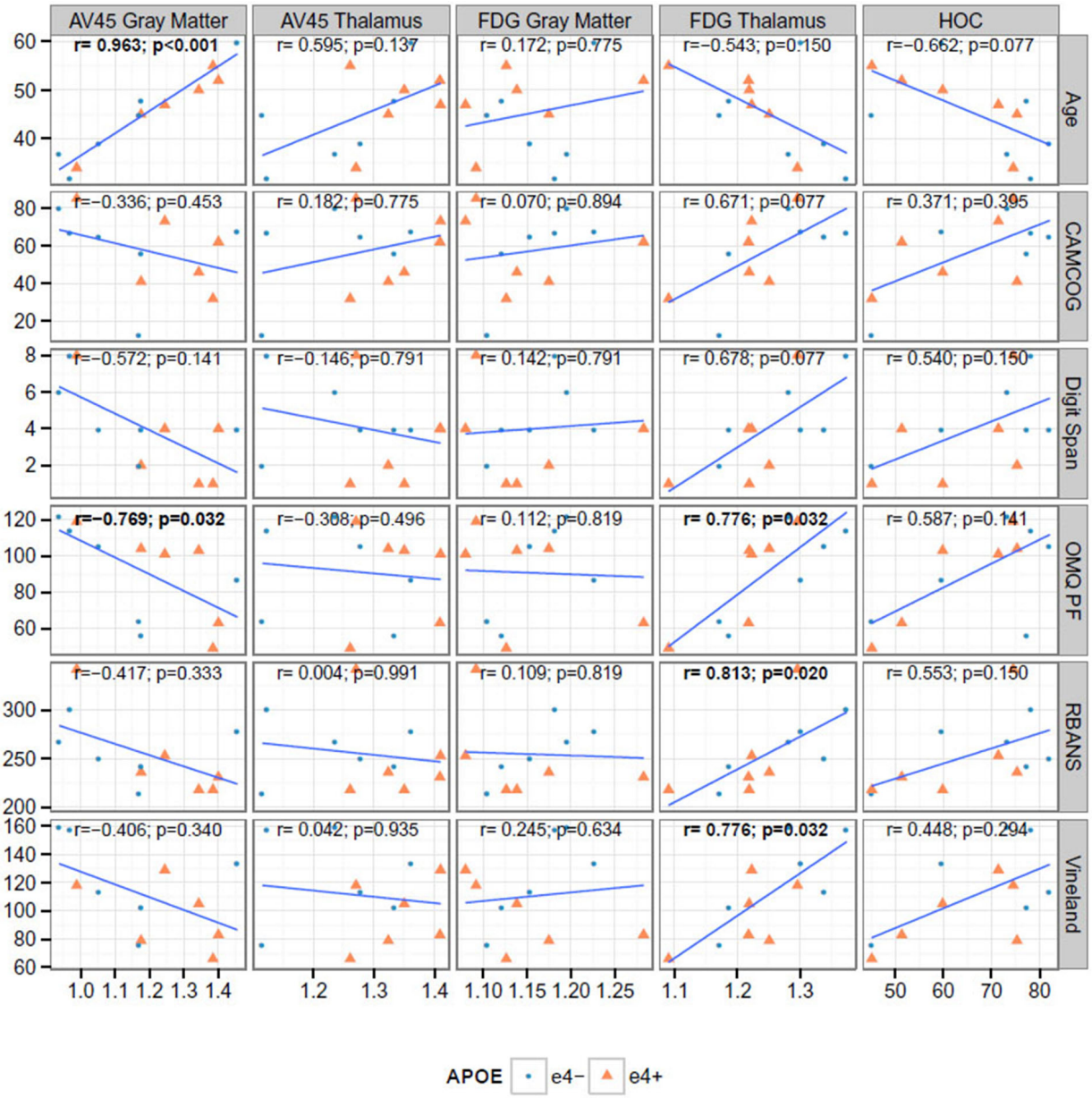

FIGURE 2 | Correlations between cognitive and neuroimaging measures. The bold text indicates Spearman rank correlations (*r*) that are significant at the 0.05 level after false discovery rate adjustment. AV45 = ^{18}F-florbetapir.

and with age or cognition (**Figure 3**); nor between MRI and PET (**Figure 4**). We did find a significant correlation between A beta 42 and age (r = 0.602, p = 0.038).

Correlation Between PET measures and HOC

We found a significant negative correlation between Florbetapir (AV45) uptake in gray matter and HOC (r = −0.615, p = 0.037). FDG uptake in thalamus and HOC were positively correlated (r = 0.671, p = 0.020) see **Figure 4**.

Retinal Amyloid Imaging

We imaged amyloid plaques in the retina of all subjects in this small cohort, **Figure 5**. All subjects demonstrated amyloid positivity.

Discussion

Although limited in sample size, this small pilot study provides strong support for the feasibility of a multicenter longitudinal AD biomarker study in adults with DS. Our findings also show that prior to dementia onset, changes in volumetric MRI,

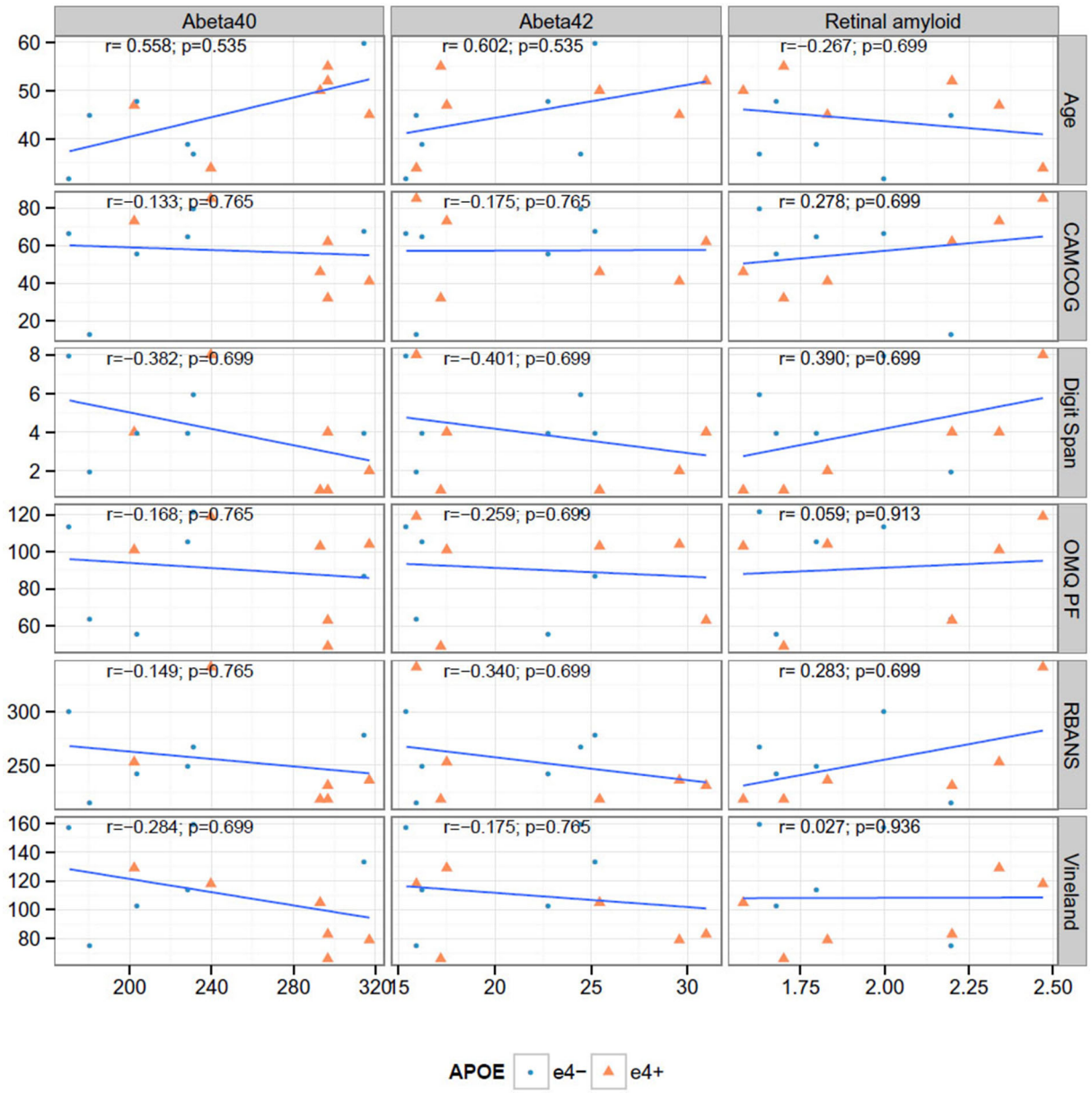

FIGURE 3 | Correlations between cognitive measures, retinal amyloid and plasma biomarkers. None of the Spearman rank correlations (*r*) are significant at the 0.05 level after false discovery rate adjustment.

amyloid PET and FDG PET and plasma are detectable and consistent with preclinical AD in adults with DS. Adults with DS had elevated levels of plasma Aβ1–42 concentrations and plasma Aβ1–42:Aβ1–40 ratios. These findings are consistent with previously published findings for individuals with DS (Schupf et al., 2010). Consistent with previous autopsy studies, most subjects demonstrated amyloid PET positivity reflecting fibrillar amyloid plaque deposition.

We also find adults with DS can tolerate amyloid-β deposition without significant effects on cognitive functioning. This has been reported by others (Hartley et al., 2014) and likely reperesents the preclinical stage of AD.

Study Strengths

We successfully studied AD biomarkers in all participants with DS, who, in the absence of an effective prevention treatment, are certain to develop symptoms of AD. With this cohort, we confirm feasibility of a large-scale multicenter longitudinal study designed to characterize trajectories of cognitive decline. The fact that DS has native wild-type APP may make it more relevant to studying biomarkers applicable to the general sporadic AD. Additionally, we compared several different brain imaging and fluid biomarker measurements, as well as exploratory biomarkers such as retinal Aβ imaging, to characterize some of the earliest biomarker changes associated with the predisposition to AD.

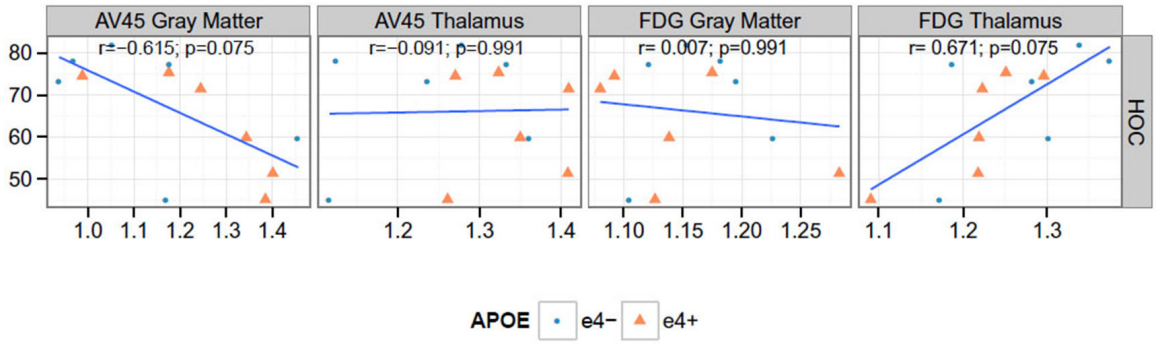

FIGURE 4 | Correlations between PET and hippocampal occupancy (HOC). None of the Spearman rank correlations (*r*) are significant at the 0.05 level after false discovery rate adjustment. AV45 = ^{18}F-florbetapir.

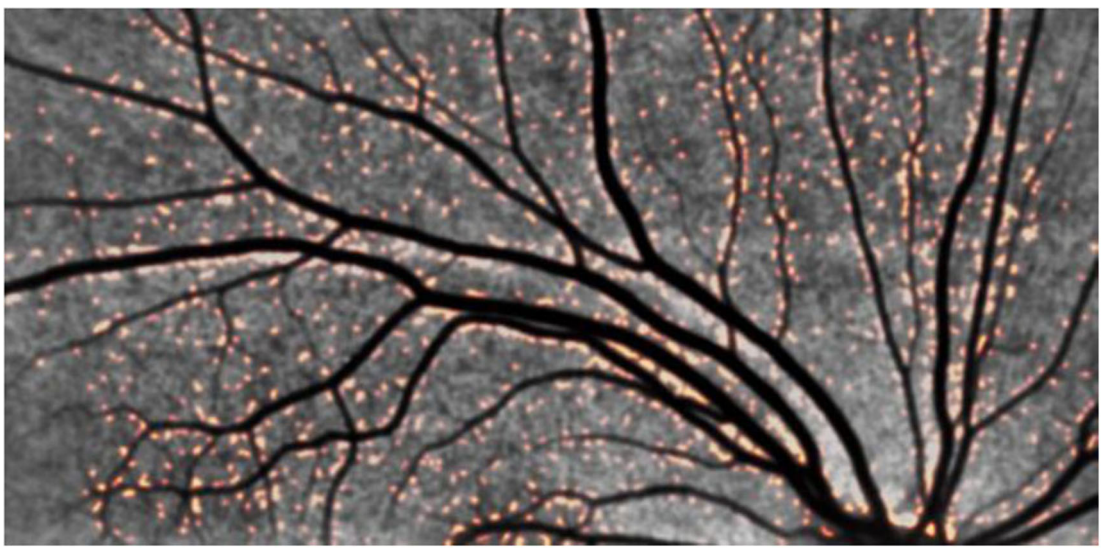

FIGURE 5 | Representative retinal images from an adult with DS demonstrating positive amyloid plaques in DS. Note the orange-colored puncta. The distribution in the vicinity of blood vessels is striking, pointing to a retinal manifestation of congophilic angiopathy.

Limitations and Issues of Interpretation

This study also has several limitations, including small sample size, absence of longitudinal data, and uncertainty in the extent to which our findings are generalizable to other causes of late-onset AD. Although the retinal amyloid findings should be regarded as exploratory, the uncorrected significance levels, bilateral pattern, and resemblance to the pattern reported previously in patients with AD reduce the likelihood that they are attributable to the type I error associated with multiple regional comparisons. Although our findings are currently limited to DSBI pilot participants, we have sought to harmonize our biomarker measurements and undertake biological fluid assays in the same laboratory used by investigators in the study of other DS cohorts (LonDowns and Fundació Catalana de Síndrome de Down), thus providing complementary data and converging evidence in the preclinical study of AD in DS patients.

Additional studies are needed to clarify several issues: the extent to which the structural and functional abnormalities identified in young adults with DS at genetic risk for AD precede Aβ plaque deposition; whether these changes are neurodegenerative or developmental; whether or not there is any cerebral fibrillar Aβ deposition in young adults with DS.

Conclusion

Adults with DS have volumetric MRI, Aβ PET, FDG PET and retinal Aβ changes, along with plasma biomarker findings consistent with Aβ1–42 overproduction. This study shows some of the earliest known AD biomarker changes in adults with DS and underscores the need for studies to clarify the earliest brain changes associated with the predisposition to AD. We have recently added Tau PET imaging to the set of biomarkers assessed in this cohort. Under the auspices of the DSBI pilot, we are continuing to characterize the

age-related trajectory of biomarker changes associated with preclinical AD to set the stage for the first clinical trial of an anti-Aβ therapy in the preclinical treatment of AD in adults with DS.

Author Contributions

MR, PSA, SN, RR and WM designed the study. MR, HW, JB, MD and RR executed the study, performed the research, analyzed the data and wrote the manuscript.

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Dissecting Alzheimer disease in Down syndrome using mouse models

Xun Yu Choong[1,2], *Justin L. Tosh*[1,2], *Laura J. Pulford*[1,2] *and Elizabeth M. C. Fisher*[1,2]*

[1] *Department of Neurodegenerative Disease, Institute of Neurology, University College London, London, UK,* [2] *The LonDownS Consortium, London, UK*

Down syndrome (DS) is a common genetic condition caused by the presence of three copies of chromosome 21 (trisomy 21). This greatly increases the risk of Alzheimer disease (AD), but although virtually all people with DS have AD neuropathology by 40 years of age, not all develop dementia. To dissect the genetic contribution of trisomy 21 to DS phenotypes including those relevant to AD, a range of DS mouse models has been generated which are trisomic for chromosome segments syntenic to human chromosome 21. Here, we consider key characteristics of human AD in DS (AD-DS), and our current state of knowledge on related phenotypes in AD and DS mouse models. We go on to review important features needed in future models of AD-DS, to understand this type of dementia and so highlight pathogenic mechanisms relevant to all populations at risk of AD.

Keywords: Alzheimer disease, APP, Down syndrome, mouse models, trisomy 21

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***Correspondence:**
Elizabeth M. C. Fisher, Department of Neurodegenerative Disease, UCL Institute of Neurology, Queen Square, London WC1N 3BG, UK
e.fisher@prion.ucl.ac.uk

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Introduction: AD-DS, the Most Common Genetic Form of AD

Down syndrome (DS) is a complex, heterogeneous disorder caused by the presence of an extra copy of human chromosome 21. Trisomy 21 is a common condition, with an incidence of 1 in 750 live births (Parker et al., 2010). Prevalence in many countries is growing due to increasing maternal age, the greatest risk factor for DS (Loane et al., 2013), together with rises in DS life expectancy (Yang et al., 2002; Bittles and Glasson, 2004). In Northern Europe, for example, the number of people aged over 40 years with DS is approximately double what it was in 1990, and in the UK this age group accounts for a third of the estimated 40,000 people with DS (Wu and Morris, 2013).

The clinical presentation of DS varies extensively and includes features present in all individuals, such as cognitive deficits, and those seen in only some people, such as heart defects (Zigman, 2013; Jensen and Bulova, 2014). Alzheimer disease (AD) pathology is found in the brains of virtually all people with DS by 40 years of age (Wisniewski et al., 1985; Mann and Esiri, 1989), and trisomy 21 causes an increased risk of dementia such that approximately one third of the DS population has AD ("AD-DS") by the age of 60, with an estimated lifetime prevalence of 90% for all people with DS (Prasher and Krishnan, 1993; Holland et al., 1998; Coppus et al., 2006; Margallo-Lana et al., 2007; McCarron et al., 2014). However, while AD-DS is one of the largest contributors to morbidity and mortality in DS (Coppus et al., 2008), not all individuals develop dementia, even by 70 years of age (Krinsky-McHale et al., 2008; Ghezzo et al., 2014). Thus, the DS population has the most common genetic form of early-onset AD, caused by trisomy 21. Studying AD-DS allows investigation of the initial pathogenic events leading to AD and the development of dementia, relevant to both people with DS and to the general population.

One approach to dissecting human disease is through studying mouse models, and a large number of transgenic strains have been generated to understand specific aspects of AD

pathology, most of which have human gene mutations that give rise to rare early-onset familial Alzheimer disease (FAD; Braidy et al., 2012; Webster et al., 2014). In the last decade, chromosome engineering techniques have enabled the generation of an array of DS mouse models that will allow us to dissect the genetic contribution of chromosome 21 (Hsa21), or regions of the mouse genome syntenic to Hsa21, to DS phenotypes. These models recapitulate a wide range of DS features, including neurobiological, behavioral and aging-related aspects (Zhang et al., 2012b; Ruparelia et al., 2013). Thus, in the study of AD-DS, mouse models of DS offer an increasingly important approach to understanding pathogenic mechanisms, so informing us about pathways and networks relevant to all populations at risk of dementia.

Here, we present an overview of clinical features of AD-DS, compared to other genetic forms of AD, to highlight human phenotypes that may be assessed in mechanistic studies of mouse models. We then give examples of data from DS mouse models compared to transgenic mice modeling aspects of AD pathology, to illustrate informative findings from both types of model. We also offer examples of potentially helpful data for investigating AD-DS from the outcomes of overexpressing single genes from Hsa21. Finally, we consider the important features for mouse models to enhance our understanding of AD-DS, and therefore the pathogenetic mechanisms relevant to all AD. For brevity, citations may not necessarily be the original papers, but useful reviews or later references.

Genetic Forms of AD, Including AD-DS

The *APP* gene lies on Hsa21 and encodes the amyloid precursor protein that is at the heart of the amyloid cascade hypothesis of Alzheimer disease (Glenner and Wong, 1984; Hardy and Higgins, 1992; Hardy and Selkoe, 2002). This hypothesis was generated partly from the observation that extracellular plaques in brains of people with AD are composed of Aβ peptides that are products of APP metabolism. The hypothesis suggests that abnormal APP metabolism initiates AD pathogenesis by triggering a set of events that result in Aβ aggregation, particularly of the Aβ42 peptide, in these extracellular plaques. This leads to the formation of intracellular neurofibrillary tangles, primarily composed of the protein tau, and eventually loss of synapses and neurons. The relationship between the histopathological features of AD and dementia is not yet clear (Castellani and Perry, 2014).

The amyloid cascade hypothesis is currently the most widely-accepted paradigm guiding investigations of AD pathogenesis, and is supported at least in part by the rare cases of FAD caused by different mutations in *APP*, and in the presenilin genes *PSEN1* and *PSEN2* that affect APP processing. *APP* mutations may, for example, result in an increase in total Aβ production, or a relative increase in Aβ species associated with pathogenicity (Ryan and Rossor, 2010).

Importantly for understanding AD-DS, the link between *APP* and AD also extends to gene dose: in rare forms of FAD, duplication of the wildtype *APP* locus alone ("Dup-APP") is sufficient to cause highly penetrant early-onset AD (Rovelet-Lecrux et al., 2006; Sleegers et al., 2006). Dup-APP cases demonstrate that the three doses of *APP* arising from trisomy 21 are likely to be causative for AD-DS. Conversely, although very rare, partial trisomy 21 excluding *APP* (i.e., with two "doses" of *APP*) does not appear to lead to AD (Prasher et al., 1998; Korbel et al., 2009).

While people with DS and Dup-APP are at high risk of dementia, presumably in both cases because of *APP* triplication, there are some intriguing differences in their AD-related clinical features (Wiseman et al., 2015). Examining the effects of different *APP* genotypes may therefore provide insights into the modulation of *APP* pathogenesis. **Table 1** shows key examples of phenotypes in AD-DS and how these compare with Dup-APP, FAD due to other *APP* mutations (primarily point mutations) and late-onset sporadic AD (SAD). Mutations in *PSEN1* and *PSEN2*, which do not map to Hsa21, are not included.

However, a difficulty in analysing phenotypes is the considerable heterogeneity in clinical presentation within each *APP* genotype, even within families with the same mutation. For example, there is a wide variety of non-cognitive symptoms and behavioral changes across all four AD genotypes, including personality changes (Nelson et al., 2001; Ball et al., 2008), hallucinations (Sleegers et al., 2006; Basun et al., 2008; Guyant-Marechal et al., 2008), paranoia (Sleegers et al., 2006; Pilotto et al., 2013), and delusions (Burns et al., 1990), some of which are associated with cognitive decline (Adams and Oliver, 2010). Another important issue in diagnosing AD in AD-DS is that dementia is an additional cognitive deficit acquired on top of the baseline cognitive impairment found in people with DS: distinguishing between cognitive deficits due to intellectual disability, and decline at early stages of AD, is therefore an important challenge. However, diagnosis of dementia by experienced clinicians has been shown to be accurate in DS, and even more reliable than recent operational dementia criteria (Sheehan et al., 2015). Further, a few clinical features stand out in AD-DS—a striking example, albeit one of unknown relevance to AD, is seizure susceptibility in adulthood, which appears heightened by *APP* duplication, as both AD-DS (84%) and Dup-APP (57%) have significantly higher rates of seizures than SAD (10–20%). This may indicate specific pathways that are progressively disrupted by *APP* duplication, resulting in damaging electrical activity in the brain.

Dup-APP and FAD caused by *APP* mutations are relatively rare, and much information about these conditions remains to be gathered, for example, on synaptic dysfunction, oxidative stress and neuroinflammation. In contrast, AD-DS arises in a population with a well-defined genetic basis and a sizeable prevalence, which means it is of great value for investigating AD pathogenesis for everyone at risk of dementia.

Modeling DS, Including AD-DS, in Mice

Human chromosome 21 has synteny with the mouse genome, such that its ortholog genes are found in three blocks with conserved order and gene orientation on mouse chromosomes 10 (Mmu10), Mmu16, and Mmu17 (Hattori et al., 2000; Dierssen et al., 2009); the mouse *App* gene lies on Mmu16 (**Figure 1**).

TABLE 1 | Comparison of phenotypes from different genetic forms of human Alzheimer disease.

Phenotype		AD-DS: three copies of wildtype *APP*	FAD (Dup-APP): three copies of wildtype *APP*	FAD (*APP* mutations): Usually heterozygous for a mutant *APP* allele. ***N.B. these mutations do not necessarily act by the same mechanisms***	SAD: two copies of wildtype *APP*
CLINICAL SYMPTOMS					
Cognition	Incidence and age of onset of dementia	Less than 40 years of age, <5% people with DS have dementia but prevalence doubles every 5 years; by 55–60 years, 50–70% of DS have AD (Tyrrell et al., 2001; Hartley et al., 2015) Total prevalence across lifespan estimated at ∼90% (McCarron et al., 2014)	Dementia onset ∼42–59 years of age (Cabrejo et al., 2006)	Dementia onset ∼45–60 years of age (Ryan and Rossor, 2010)	Dementia onset usually >65 years of age (Querfurth and LaFerla, 2010)
	Pre-clinical cognitive symptoms	Pre-existing cognitive impairments complicate diagnosis of AD in DS (Zigman, 2013) Memory deficits may occur up to 3 years before dementia diagnosis (Krinsky-McHale et al., 2002)	No apparent pre-symptomatic cognitive impairment (Cabrejo et al., 2006; Rovelet-Lecrux et al., 2006)	Pre-symptomatic impairment of verbal memory and IQ; early progressive impairment of episodic memory (Rovelet-Lecrux et al., 2006; Hooli et al., 2012)	Mild cognitive impairment (cognitive symptoms, notably memory problems, which do not significantly affect function) precedes dementia (Albert et al., 2011), although only 5–20% go on to develop dementia
	Clinical presentation of dementia	Amnestic presentation similar to AD after taking into account pre-existing baseline intellectual deficits However, changes in behavior and personality are more common than SAD (Krinsky-McHale et al., 2000; Devenny et al., 2002; Ball et al., 2008)	Slow and progressive memory impairment and loss of cognition (Sleegers et al., 2006)	Most cases have similar amnestic presentation to SAD (Pilotto et al., 2013)	Progressive deficits in episodic memory, semantic knowledge, working memory, and attention (Weintraub et al., 2012)
	Sex differences	No difference between sexes (Coppus et al., 2006)	Not reported	Not reported	Women at higher risk (Musicco, 2009)
Epilepsy		Up to 84% AD-DS experience seizures (Mendez and Lim, 2003; De Simone et al., 2010)	Up to 57% exhibit seizures (Rovelet-Lecrux et al., 2006)	Seizures described in at least four different APP mutations (Kumar-Singh et al., 2000; Murrell et al., 2000; Grabowski et al., 2001; Pasalar et al., 2002)	Up to 10–20% of patients exhibit seizures (Mendez and Lim, 2003; Palop, 2009)
CLASSICAL AD NEUROPATHOLOGY: Aβ AND TAU					
Aβ accumulation and deposition	Intracellular Aβ	Intraneuronal accumulation of Aβ42 has been seen at 3 years of age. Levels decline as diffuse and dense core plaques develop (Mori et al., 2002)	Intraneuronal accumulation of Aβ40 in post mortem brain. No intraneuronal Aβ42 detected (Cabrejo et al., 2006)	Not reported	Intracellular staining found in post mortem SAD tissue (LaFerla et al., 2007)

(Continued)

TABLE 1 | Continued

Phenotype		AD-DS: three copies of wildtype *APP*	FAD (Dup-APP): three copies of wildtype *APP*	FAD (*APP* mutations): Usually heterozygous for a mutant *APP* allele. *N.B. these mutations do not necessarily act by the same mechanisms*	SAD: two copies of wildtype *APP*
	Extracellular Aβ	Earliest extracellular deposition found at 8 years of age (Leverenz and Raskind, 1998) Aβ40 undetectable in plaques in DS brain <50 years of age. Proportion of Aβ40 in plaques gradually increases until =50 years of age 42% of dense-core plaques comprise of Aβ40 (Iwatsubo et al., 1995) Amyloid plaques universal in DS people by age 31 (Leverenz and Raskind, 1998; Hartley et al., 2015)	Parenchymal lesions predominantly composed of Aβ42. Vascular amyloid predominantly Aβ40 (Cabrejo et al., 2006; Rovelet-Lecrux et al., 2006) Abundant parenchymal and vascular lesions as both dense-core and diffuse plaques (Cabrejo et al., 2006; Guyant-Marechal et al., 2008)	Increased Aβ42/Aβ40 ratio and/or increased Aβ production (Tanzi, 2012). Rare APP A673T mutant confers protection against AD pathology (Peacock et al., 1993; Hashimoto and Matsuoka, 2014) Pattern and progression of amyloid plaque deposition is largely identical to SAD. However, mutations within the Aβ sequence can cause increased deposition in the vasculature (Pilotto et al., 2013)	Accumulation of Aβ42 and Aβ40 into amyloid plaques. Aβ42 is more abundant in plaques (Serrano-Pozo et al., 2011) Amyloid plaque deposition progresses in a stereotypical fashion characterized by Thal phases I-V (Thal et al., 2002) Highest accumulation of plaques found in layers II–IV of the isocortex (Braak and Braak, 1991; Serrano-Pozo et al., 2011)
	Cerebral Amyloid Angiopathy (CAA) and Intra-cranial Hemorrhage (ICH)	CAA pathology common in DS. ICH is rare (Mann, 1988a; McCarron et al., 1998; Naito et al., 2008)	CAA is ubiquitous (Cabrejo et al., 2006; Sleegers et al., 2006; Rovelet-Lecrux et al., 2007; Kasuga et al., 2009) ICH in 20–50% of cases (Cabrejo et al., 2006; Rovelet-Lecrux et al., 2007; Guyant-Marechal et al., 2008; Kasuga et al., 2009)	CAA is in a large number of FAD mutations but not all (Ryan and Rossor, 2010) Arctic and Dutch APP mutations both affect residue 693 but only patients with Dutch mutation develop CAA and ICH (Basun et al., 2008; Ryan and Rossor, 2010)	∼50–80% of cases have CAA, deposits primarily composed of Aβ40 (Jellinger et al., 2007; Serrano-Pozo et al., 2011) ICH in ∼3% of SAD cases, possibly related to hypertension (Jellinger et al., 2007)
Neurofibrillary tangles		NFTs present in almost all people with DS by age 45. Density of NFTs triples between age 40–50 (Wisniewski et al., 1985; Goedert et al., 1992) NFT density correlates more strongly with clinical dementia rating than Aβ plaque count (Margallo-Lana et al., 2007) NFTs only manifest subsequent to dense-core amyloid plaques (Hartley et al., 2015)	NFTs consistent with late stage AD present at time of death (Rovelet-Lecrux et al., 2006)	Different FAD mutations exert highly variable effects on NFTs, from absence of NFTs in Arctic mutations to severe pathology (Ryan and Rossor, 2010)	Stereotypical spatiotemporal progression of NFTs begins in the allocortex of the medial temporal lobe with six stages of development, distinguished by Braak stages (Braak and Braak, 1991) Increased levels of total tau and phospho-tau correlate with increase in SAD severity (Wallin et al., 2006; Serrano-Pozo et al., 2011)

(Continued)

TABLE 1 | Continued

Phenotype	AD-DS: three copies of wildtype *APP*	FAD (Dup-APP): three copies of wildtype *APP*	FAD (*APP* mutations): Usually heterozygous for a mutant *APP* allele. ***N.B. these mutations do not necessarily act by the same mechanisms***	SAD: two copies of wildtype *APP*
Neuronal loss and brain atrophy	Neuronal atrophy follows SAD pattern but trend for less relative cell loss and atrophy compared to SAD (Mann, 1988b) Selective loss of BFCNs from as early as 5.5 months of age. Progressive loss of neurons in the Nucleus basalis of Meynert during aging (Casanova et al., 1985; McGeer et al., 1985)	Diffuse cortical atrophy with parietal dominance and neuronal loss (Cabrejo et al., 2006; Sleegers et al., 2006; Rovelet-Lecrux et al., 2007; Guyant-Marechal et al., 2008; Kasuga et al., 2009)	Similar neuronal atrophy pattern to SAD with a slightly more severe medial-temporal pattern (Pilotto et al., 2013)	Characteristic loss of neurons and white matter (Querfurth and LaFerla, 2010). Neuronal loss correlates with NFTs (Gómez-Isla et al., 1997; Serrano-Pozo et al., 2011) Basal forebrain atrophy correlates with Aβ burden (Kerbler et al., 2015)
OTHER FEATURES OF AD PATHOLOGY				
Synaptic loss and dysfunction	Synaptic protein expression decreased in aging DS brain (Downes et al., 2008) GABA levels decreased in post-mortem hippocampus and temporal cortex (Reynolds and Warner, 1988; Seidl et al., 2001; Martínez-Cué et al., 2014)	Not reported	Not reported	Synapse loss is best correlate of cognitive decline and precedes neuronal loss (Ingelsson et al., 2004; Scheff et al., 2007) GABA significantly reduced in post mortem cortical but not subcortical brain regions. *In vivo* evidence of GABA loss in parietal cortex (Seidl et al., 2001; Bai et al., 2014)
Oxidative stress and proteostasis	Some proteins oxidatively modified differently in DS and control groups, suggesting DS subjects vulnerable to oxidative damage (Di Domenico et al., 2014)	Not reported	Not reported	Increased levels of oxidative stress are a hallmark of SAD pathology and linked to aging (Madeo, 2013)
Endosomal dysfunction	Endosome enlargement, alterations in morphology and function in young DS (pre-AD) and DS fibroblasts (Jiang et al., 2010)	Not reported	Enlarged endosomes modulated by ApoE status (Cataldo et al., 2001)	Enlarged endosomes detected in preclinical stages (Cataldo et al., 1997, 2000) Aβ accumulates within late endosomes in AD brain (Takahashi et al., 2002)

(Continued)

TABLE 1 | Continued

Phenotype	AD-DS: three copies of wildtype *APP*	FAD (Dup-APP): three copies of wildtype *APP*	FAD (*APP* mutations): Usually heterozygous for a mutant *APP* allele. ***N.B. these mutations do not necessarily act by the same mechanisms***	**SAD: two copies of wildtype *APP***
Neuroinflammation	Dystrophic microglia and absence of activated microglia at 40 years of age, coincident with tau pathology (Xue and Streit, 2011) Increased astrocytic activation in early DS, increases with age and correlates with amyloid deposition (Royston et al., 1999)	Not reported	Not reported	Hyper-reactive, dystrophic microglia associated with dense-core plaques and NFTs (McGeer et al., 1987; Streit et al., 2009) Reactive astrocytes locate early to dense-core plaques, triggered by Aβ (Itagaki et al., 1989; Pike et al., 1995) Higher neuroinflammation in younger (<80) compared to older patients with SAD, suggesting importance in early stages of disease (Hoozemans et al., 2011)

Down syndrome (AD-DS), familial AD due to APP duplications (Dup-APP), familial AD due to APP mutations (FAD), and sporadic Alzheimer disease (SAD) Abbreviations: BFCNs, basal forebrain cholinergic neurons; CAA, cerebral amyloid angiopathy; GABA, γ-Aminobutyric acid; ICH, intra-cranial hemorrhage; ID, intellectual disability; NFT, neurofibrillary tangles.

Mice with precisely-defined trisomies (or monosomies) have been generated, now usually by chromosome engineering (Brault et al., 2006; Tybulewicz and Fisher, 2006), to provide a set of models that are segmentally trisomic for regions orthologous to Hsa21 (Davisson et al., 1993; Sago et al., 1998; Olson et al., 2004; Li et al., 2007; Herault et al., 2009; Pereira et al., 2009; Yu et al., 2010a; Liu et al., 2011, 2014; Brault et al., 2015).

Generating many models with different partial trisomies creates a mapping panel in which individual phenotypes may be assessed in several strains, and so assigned to specific trisomic chromosomal region(s). As all DS phenotypes presumably arise from abnormal gene dosage, candidate genes that when present in three copies give rise to all or part of the phenotype, can be chosen from the trisomic critical region. Individual candidate genes can then be studied, for example, in overexpression or knockout models, to assess the effects of different copy numbers of the gene. **Figure 1** is an overview of DS mouse models and the chromosomal segments for which they are trisomic. **Table 2** details the gene content for each DS mouse model shown, including protein-coding and non-protein-coding genes relevant to human trisomy 21.

The most complete mouse model to date, *Dp(10)1Yey/+;Dp(16)1Yey/+;Dp(17)1Yey/+*, is trisomic for all Hsa21 syntenic regions and was generated by crossing three DS mouse models, each carrying duplications of the respective Hsa21 orthologous regions on Mmu10, Mmu16 and Mmu17 (Li et al., 2007; Yu et al., 2010a,b; **Figure 1**). However, the vast majority of studies relating to AD-DS have been performed on the Ts65Dn mouse, as this has been an extremely important "standard model" of DS for many years, prior to the development of newer strains by chromosome engineering (Davisson et al., 1993; Reeves et al., 1995; **Table 2**). The Ts65Dn mouse carries a Robertsonian translocation resulting in trisomy of ∼42% of the protein-coding genes orthologous to Hsa21, but it also has 79 additional genes (including long non-coding sequences) from Mmu17 that are outside the Hsa21 region of synteny, and these need to be taken into account when analysing phenotypes (Duchon et al., 2011; Reinholdt et al., 2011). These extra triplicated genes that do not relate to DS happen to include non-Hsa21 genes, such as *SYNJ2* and *TIAM2* that have Hsa21/Mmu16 paralogues (*SYNJ1*, *TIAM1*), which may complicate phenotype-genotype correlations (Duchon et al., 2011). Other triplicated genes in Ts65Dn irrelevant to DS include several genes encoding dynein light chains that may influence endosomal trafficking, and so potentially affect neuronal phenotypes (Hartley et al., 2015).

A different type of mouse model of DS is the "humanized" transchromosomic "Tc1" mouse that carries a freely-segregating Hsa21 (O'Doherty et al., 2005), which is functionally trisomic for ∼75% of Hsa21 protein-coding genes (Gribble et al., 2013). However, this extra chromosome is rearranged, and lost stochastically at different rates in different mouse tissues—thus, Tc1 mice are mosaic for the human chromosome. With respect to AD research, the *APP* gene is not functionally trisomic in Tc1 mice because of a rearrangement that has occurred by chance, so this animal expresses just the two endogenous copies of mouse *App* (Sheppard et al., 2012).

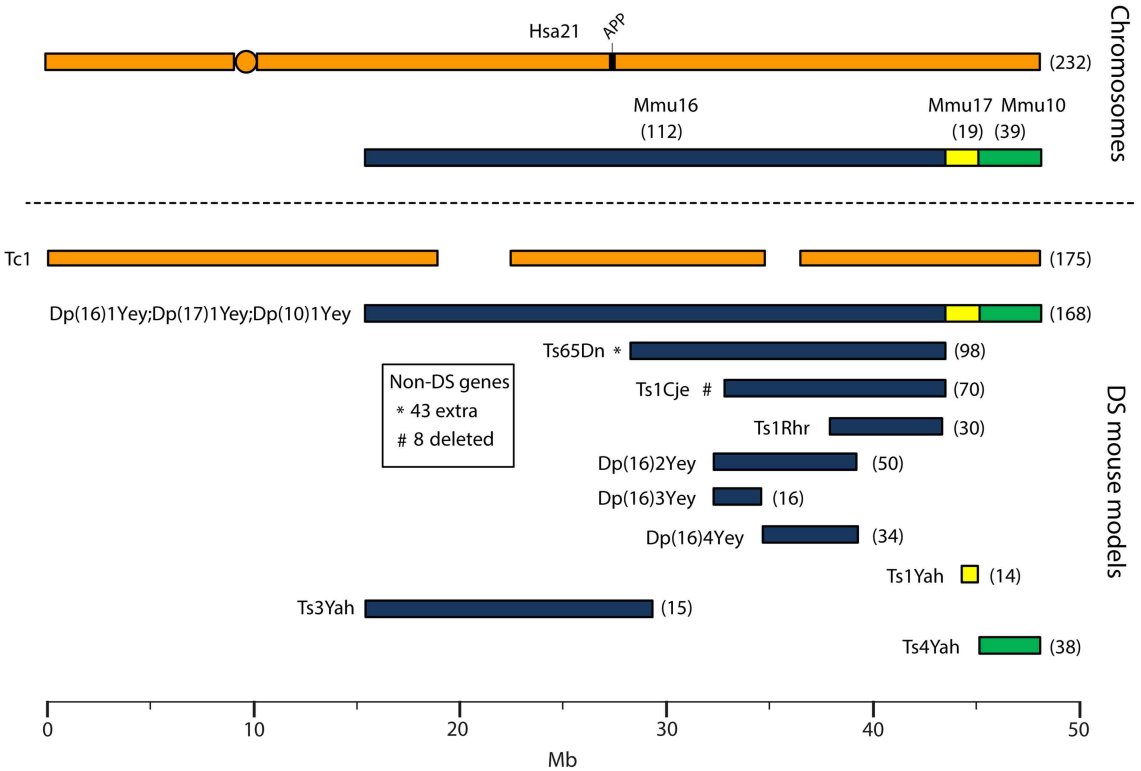

FIGURE 1 | Human chromosome 21 (Hsa21), orthologous mouse chromosomes (Mmu), and key mouse models of Down syndrome. Diagram representing Hsa21 and its alignment with syntenic regions on Mmus 16, 17, and 10. The orange circle represents the human centromere and mouse models are color-coded and aligned according to the chromosomal segment for which they are trisomic. Numbers in brackets represent the number of protein-coding Hsa21 orthologous genes within each region or mouse model, according to Ensembl release 79 and the breakpoints published in papers referenced here. The Tc1 mouse is the only model which carries Hsa21, though genomic rearrangements and deletions (indicated by breaks in the chromosome) mean the mouse is functionally trisomic for only ~75% of Hsa21 genes (Gribble et al., 2013). All other mouse models carry duplications of mouse orthologues. The Dp1(16)Yey;Dp1(17)Yey;Dp1(10)Yey (or Ts1Yey;Ts3Yey;Ts2Yey) mouse was generated by crossing together three partial trisomy models (Yu et al., 2010a) and spans the entirety of the Hsa21-syntenic regions. The Ts65Dn mouse (Davisson et al., 1993) contains a freely segregating segment of Mmu16, however it is also trisomic for 43 extra protein-coding genes on the centromeric section of Mmu17 that are not relevant to DS (indicated by an asterisk (*) and accompanying text box; Duchon et al., 2011; Reinholdt et al., 2011). The Ts1Cje mouse (Sago et al., 1998) also contains a monosomy of eight protein-coding genes on Mmu12, irrelevant to the DS phenotype (indicated by "#" and accompanying text box. Gene numbers are based on Ensembl release 79, compared to the original seven monosomic genes detailed in Duchon et al., 2011). Other mice are Ts1Rhr or Dp1(16)Rhr mice (Olson et al., 2004); Ts1Yah mice (Pereira et al., 2009); Ts3Yah (previously published as Ts2Yah; Brault et al., 2015); and Ts4Yah mice (previously published as Ts3Yah mice; Herault et al., 2009). Other useful examples of mouse models include the Ts43H model (not shown) which is partially trisomic for Mmu17 including some genes with ortholog on Hsa21 (Vacík et al., 2005). The scale is in megabase pairs (Mb).

While many DS mouse models have been published, there is no single complete model, and the usefulness of these strains lies in their comparative and complementary use in studying genotype-phenotype relationships, including AD-related phenotypes (**Table 3**). These studies enable us to map critical dosage-sensitive genes because each locus is likely expressed at trisomic levels, mimicking human DS transcription. We can also study the interactions of Hsa21 dosage-sensitive genes with the rest of the genome (Hsa21 and non-Hsa21), as well as effects exerted by aneuploidy *per se*.

Modeling Amyloid Deposition in Mice

In contrast to the segmental duplication of tens of endogenous wildtype genes in DS mouse strains, AD models are primarily transgenic lines that overexpress one or more of the human mutant genes that cause FAD. These transgenes usually insert at random sites in the genome and may be driven by artificial promoters (see examples in **Table 4**), which vary in terms of their spatial and temporal expression patterns, and result in expression at often 5–10 fold compared to endogenous mouse orthologue (Balducci and Forloni, 2011; Hall and Roberson, 2012). Overexpressing wildtype human *APP* or mouse *App* does not result in amyloid deposition (Elder et al., 2010); hence the need to use known AD-causative mutant sequences in transgenic mice.

In general, while mutant *APP* transgenic mice develop robust amyloid deposition, synaptotoxic features and memory impairments, none of them reproduces tau-containing neurofibrillary tangles, the hallmark pathology of AD which most

TABLE 2 | Trisomic region and triplicated gene content in Down syndrome mouse models shown in Figure 1 compared with Hsa21 (Ensembl release 79).

		Protein-coding genes		Non-protein-coding genes		Total genes		% Protein-coding genes from Hsa21
	Hsa21	232		648		880		
DS mouse model	Official MGI name*	Mouse genes	Hsa21 genes	Mouse genes	Hsa21 genes	Mouse genes	Hsa21 genes	
Tc1	B6;129S-Tc(Hsa21)1TybEmcf/J	–	175	–	Undetermined	–	N/A	75
Dp(16)1Yey	B6.129S7-Dp(16Lipi-Zbtb21)1Yey/J	149	112	112	6	261	118	48
Dp(17)1Yey	B6;129S7-Dp(17Abcg1-Rrp1b)3Yey/J	19	18	6	0	25	18	8
Dp(10)1Yey	B6;129S7-Dp(10Prmt2-Pdxk)2Yey/J	55	39	20	1	75	40	17
Ts65Dn**	B6EiC3Sn a/A-Ts(1716)65Dn	133	98	71	3	204	101	42
Ts1Cje***	B6.Cg-T(12;16)1Cje/CjeDnJ	76	70	51	1	127	71	30
Ts1Rhr	B6.129S6-Dp(16Cbr1-Fam3b)1Rhr/J	32	30	20	0	52	30	13
Dp(16)2Yey	129-Dp(16Tiam1-Kcnj6)6Yey/J	53	50	37	1	90	51	22
Dp(16)3Yey	129-Dp(16Tiam1-Il10rb)8Yey/J	18	16	12	0	30	16	7
Dp(16)4Yey	129-Dp(16Ifnar1-Kcnj6)10Yey/J	35	34	24	1	59	35	15
Ts1Yah	B6;129P2-Dp(17Abcg1-Cbs)1Yah/Orl	15	14	4	0	19	14	6
Ts3Yah (previously Ts2Yah)	B6;129P2-Dp(16Hspa13-App)2Yah/Orl	19	15	45	5	64	20	6
Ts4Yah (previously Ts3Yah)	B6.Cg-Dp(10Prmt2-Cstb)3Yah/Orl	54	38	20	1	74	39	16
TRISOMIC/MONOSOMIC REGIONS AND GENE CONTENT IRRELEVANT TO Hsa21 AND ITS SYNTENIC REGIONS IN MICE								
Ts65Dn**	B6EiC3Sn a/A-Ts(1716)65Dn	43	–	36	–	79	–	
Ts1Cje***	B6.Cg-T(12;16)1Cje/CjeDnJ	8	–	4	–	12	–	

Mouse genome informatics site that includes the official mouse strain names www.informatics.jax.org; the shaded line shows number of Hsa21 genes.

***indicates gene content of Ts65Dn and ***indicates gene content of Ts1Cje mice.*

TABLE 3 | Examples of AD phenotypes studied in DS mouse models, and related findings in *APP* transgenic strains described in Table 4.

Phenotype	DS models		*APP* transgenic models
Cognitive deficits	Learning and memory deficits widely demonstrated, mostly in young mice (Das and Reeves, 2011) Differentiating between early cognitive impairment and neurodegeneration in old age is a challenge (Ruparelia et al., 2013). One study suggests learning deficits in Ts65Dn worsen with age, but due to lack of motivation or motor impairment rather than neurodegeneration (Sanders et al., 2009)		Working memory, episodic memory, executive function, and attention deficits in *APP* transgenic mice from young ages (3–5 months; Webster et al., 2014) Memory impairments linked to neurotoxicity as a result of Aβ oligomers (Lesné et al., 2006) or insoluble Aβ deposits (Westerman et al., 2002) Behavioral deficits deteriorate with age in some *APP* transgenic mice (Hsiao et al., 1996; Van Dam et al., 2003)
Long-term potentiation (LTP)	Hippocampal LTP deficits reported in all models trisomic for Mmu16 regions syntenic to Hsa21, apart from Ts2Yah for which no LTP data is available (Das and Reeves, 2011) LTP increased in Dp1(17)Yey and unaltered in Dp1(10)Yey (Yu et al., 2010b) LTP deficits observed in Tc1 suggest compromised entorhinal cortex input into the dentate gyrus, contributing to impaired CA3 and CA1 function (Witton et al., 2015)		LTP studies have produced often contradictory measurements within the same mouse models (Pozueta et al., 2013) Aberrant neuronal activity is a prominent feature; restoring inhibitory synaptic activity may rescue network hypersynchrony, memory deficits and early mortality (Sanchez et al., 2011; Verret et al., 2012; Stargardt et al., 2015)
Aβ accumulation and deposition	APP protein and mRNA expression	In Ts65Dn, APP protein increases to trisomic levels from 6 months in the striatum (Hunter et al., 2003), and from 10 months in cortex and hippocampus (Seo and Isacson, 2005; Contestabile et al., 2006) APP mRNA in Ts65Dn remains similar to disomic levels at 5 months but increases at 12 months (Choi et al., 2009)	*APP* transgenic mice generally overexpress human *APP* with FAD mutations at levels at least 5x endogenous mouse *App*. *APP* transgene transcription is directed by artificial promoters (**Table 4**) allowing expression in the central nervous system, usually from embryonic or early postnatal age (Crews et al., 2010; Balducci and Forloni, 2011; Hall and Roberson, 2012)
	APP metabolism	In Ts65Dn, total APP CTF levels increased in hippocampus, enriched in synaptosomes and early endosomes from 6 months (Salehi et al., 2006; Lockrow et al., 2009) In Ts65Dn no difference in Aβ42/40 ratios, low levels of larger (~115 kDa) SDS-stable Aβ oligomers (Salehi et al., 2006; Choi et al., 2009; Peng et al., 2009)	In line with the overexpression of *APP*, Aβ levels are generally overexpressed, with some models expressing FAD mutations driving an increase in Aβ42/40 ratios (Crews et al., 2010)
Tau	Neurofibrillary pathology	In aged Ts65Dn mice increased tau and reelin detected in granules in hippocampus and olfactory bulb (Kern et al., 2011) No tau neurofibrillary tangles detectable in Tc1 and Ts1Cje brains (O'Doherty et al., 2005; Shukkur et al., 2006; Sheppard et al., 2012)	*APP* transgenic mice fail to produce neurofibrillary tangles without additional mutations introduced in presenilin or tau (Kokjohn and Roher, 2009)
	Tau hyper-phosphorylation	In Ts65Dn, Ts1Cje and Tc1, increased tau phosphorylation in hippocampus and cortex at various phosphorylation sites (Shukkur et al., 2006; Liu et al., 2008; Sheppard et al., 2012) In Tc1 this was detected in old (20 months) but not young (2 months) mice (Sheppard et al., 2012) Unphosphorylated tau decreased in Ts1Cje mice (Shukkur et al., 2006)	Hyperphosphorylation of tau and its regulation have primarily been studied in *APP* transgenic mice with additional mutations in presenilin and/or tau Hyperphosphorylated tau is detectable in some *APP* transgenic mouse models (Kokjohn and Roher, 2009; Crews et al., 2010)

(Continued)

TABLE 3 | Continued

Phenotype		DS models	*APP* transgenic models
	Regulation of tau phosphorylation	Increased phosphorylation of GSK-3β in Tc1 and Ts1Cje (Shukkur et al., 2006; Sheppard et al., 2012). Increased phosphorylation of AKT in Tc1 and Ts65Dn (Siarey et al., 2006; Sheppard et al., 2012) CDK5 expression upregulated in Ts65Dn but not in Tc1 (Pollonini et al., 2008; Sheppard et al., 2012). No difference in CDK5 activators p25/p35 levels detected in both Ts1Cje and Tc1 (Shukkur et al., 2006; Sheppard et al., 2012)	
Neuronal loss and dysfunction	Loss and dysfunction of Basal Forebrain Cholinergic Neurons (BFCNs)	Reduced BFCN numbers and cell size in Ts65Dn mice from 12 months (Cooper et al., 2001; Salehi et al., 2006) ChAT activity increased in 10-month but no different from control in 19-month Ts65Dn (Contestabile et al., 2006) Distribution of cholinergic neurons in dentate gyrus altered in Ts65Dn (Cooper et al., 2001; Salehi et al., 2006) All above alterations not observed in Ts1Cje and Ts65Dn:App$^{+/+/-}$ mice, both of which are disomic for *App* (Salehi et al., 2006)	Loss of BFCNs observed in APP23 and APP(V717I; Choi et al., 2013). No loss of BFCNs observed in APP23 (Boncristiano et al., 2002) and Tg2576 (Apelt et al., 2002) Decreased ChAT and AChE activity in basal forebrain nuclei of APP23 (Van Dam et al., 2005)
	Loss and dysfunction of noradrenergic neurons	Degenerative morphology and loss of noradrenergic neurons in rostral LC in Ts65Dn at 12 months but not 4 months (Lockrow et al., 2011b; Fortress et al., 2015)	Noradrenaline levels declined with aging in TgCRND8 hippocampus (Francis et al., 2012). No overt cell loss in LC in old APP23 and PDAPP mice, although neurons decreased in size in PDAPP (Szot et al., 2009; Francis et al., 2012)
OTHER FEATURES POTENTIALLY RELEVANT TO AD			
Epilepsy	5–10x increased rates of audiogenic seizures and seizure-related death in 21-day old Ts65Dn mice, attenuated by mGluR5 antagonists (Westmark et al., 2010)		Epileptiform activity and spontaneous non-convulsive seizures frequently observed in *APP* transgenic mice, from young ages (Born, 2015). Whether this is caused by overproduction of Aβ (Palop, 2009) or is an artifact of *APP* overexpression during development (Born et al., 2014) is unclear
Synaptic loss and dysfunction	Synaptic and dendritic abnormalities	In Ts65Dn, increased average synapse size with no change in synaptic number or density (Hernández-González et al., 2015) In Ts65Dn, dendritic spines are enlarged, less dense, and redistributed on principal neurons; arborizations are poorly developed. Similar but less severe observations in Ts1Cje (Dierssen et al., 2003; Belichenko et al., 2004) In Tc1, reduced synaptic size, complexity and density observed in hippocampus (Witton et al., 2015); decreased dendritic mushroom spines (associated with memory) at 3 months and increase in stubby spines (Haas et al., 2013) Ts1Rhr fewer thin spines (associated with learning) at 3 weeks of age (Haas et al., 2013)	Loss and alterations in dendritic spines and synapses are early features of neuronal pathology in *APP* transgenic mice models, before onset of plaque deposition and cognitive deficits. Synaptic deficits correlate well with soluble Aβ (Pozueta et al., 2013) Reduced density of mushroom-type spines of CA1 hippocampal region in two *APP* transgenic mouse models (Perez-Cruz et al., 2011)

(Continued)

TABLE 3 | Continued

Phenotype	DS models	*APP* transgenic models
Oxidative stress and proteostasis	Oxidative stress markers increased in young and old Ts65Dn mice (Lockrow et al., 2009; Shichiri et al., 2011; Di Domenico et al., 2015) Impaired mitochondrial function and increased ROS production in Ts1Cje cortical astrocyte and hippocampal neuronal cultures (Shukkur et al., 2006)	Oxidative stress increased and precedes Aβ deposition in *APP* transgenic mice. Increased Aβ levels lead to mitochondrial impairments (Eckert et al., 2010; Ye et al., 2012; Meraz-Ríos et al., 2014)
Endosomal dysfunction	Enlarged EEs in BFCNs and expression of EE proteins detected from 6 months in Ts65Dn, increasing in number with age (Cataldo et al., 2003; Salehi et al., 2006) EEs not enlarged in Ts1Cje and Ts65Dn-App$^{+/+/-}$ mice, both of which are disomic for *App* (Cataldo et al., 2003) Axonal transport disruption selectively impaired for endosomal cargo in Ts65Dn mice (Salehi et al., 2006)	No enlargement of EEs observed in APP22 and APP23 mice (Cataldo et al., 2003). Enlarged EEs found in APP23 (Choi et al., 2013) Aβ42 accumulates in endosomal compartments in Tg2576 mice before plaque deposition, and increases with age (Takahashi et al., 2002)
Neuroinflammation and glial phenotypes	Increased astrocytic protein expression and metabolic activity in old Ts65Dn mice (Holtzman et al., 1996; Contestabile et al., 2006) Increased microglial activation in basal forebrain and hippocampus of old Ts65Dn mice (Hunter et al., 2004; Lockrow et al., 2011a)	Astrocytic changes in morphology and increased calcium signaling in *APP* transgenic mice (Takano et al., 2007; Beauquis et al., 2013; Rodríguez-Arellano et al., 2015) Impairments in microglia phagocytosis and increased microglia proliferation around plaques in APP23 and Tg2576 (Frautschy et al., 1998; Krabbe et al., 2013)

Abbreviations: AChE, acetylcholinesterase; AKT, protein kinase B; BFCN, basal forebrain cholinergic neuron; CA1, Cornu Ammonis area 1; CDK5, cyclin-dependent kinase 5; ChAT, choline acetyltransferase; CTF, C-terminal fragment; EE, early endosome; GSK-3β, glycogen synthase kinase 3β; LC, locus coeruleus; LTP, long-term potentiation; mGluR5, metabotropic glutamate receptor 5; ROS, reactive oxygen species; SDS, sodium-dodecyl sulfate.

TABLE 4 | Human *APP* overexpressing transgenic mice referred to in this review (information obtained from Alzforum.org).

Mouse	Mutation	Promoter	Genetic Background	References
APP22	APP751 KM670/671NL (Swedish), V717I (London)	Human THY1	C57BL/6	Sturchler-Pierrat et al., 1997
APP23	APP751 KM670/671NL (Swedish)	Mouse Thy1	C57BL/6	Sturchler-Pierrat et al., 1997
APP(V717I)	APP695 V717I (London)	Mouse Thy1	Originally generated on FVB/N background; available at reMYND as C57BL/6xFVB/N	Moechars et al., 1999
Tg2576	APP695 KM670/671NL (Swedish)	Hamster prion protein	C57BL/6;SJL mixed background	Hsiao et al., 1996
TgCRND8	APP KM670/671NL (Swedish), V717F (Indiana)	Hamster prion protein	C3H/He-C57BL/6 mixed background	Chishti et al., 2001
PDAPP	APP V717F (Indiana)	Human PDGF	C57BL/6 x DBA2	Games et al., 1995

closely correlates with dementia (Hall and Roberson, 2012). The combined overexpression of mutant *APP* and mutant human tau is required to reproduce both amyloid and tau pathology, although these tau mutations in humans do not alone cause AD but another form of neurodegeneration, frontotemporal dementia. Mutant *APP* transgenics may be best considered models of APP/Aβ pathology (amyloid deposition) rather than full AD.

Studying AD-DS Phenotypes in Mice

In **Table 3**, we summarize examples of findings that may be informative for AD-DS from different DS (mainly Ts65Dn) mice and examples of AD models (**Table 4**). With respect to AD, a wide range of mutant *APP* transgenic strains are available in the literature, so we have chosen a few well-known examples [APP22, APP23, APP (V717I), PDAPP, Tg2576, TgCRND8] to illustrate some potential phenotypes of interest. We note that the expression of wildtype mouse APP, and wildtype or mutant human APP protein in these different models can influence amyloid pathology (Kokjohn and Roher, 2009). For example, because of amino acid differences between the two species, mouse APP may be processed with little BACE1 cleavage and so may yield three times less Aβ than wildtype human APP (De Strooper et al., 1995). In addition, the genetic background of AD mouse strains affects a range of APP/Aβ phenotypes, including plaque deposition, APP metabolism, survival, and seizure rates (Carlson et al., 1997; Lehman et al., 2003; Krezowski et al., 2004; Lassalle et al., 2008; Rustay et al., 2010; Jackson et al., 2015). Similarly, phenotypes observed in DS mice may be influenced by genetic background (O'Doherty et al., 2005; Galante et al., 2009; Costa et al., 2010; Deitz and Roper, 2011; Haydar and Reeves, 2012). We consider only *APP* transgenic models of AD, as the other genes used in such models (*PSEN1, PSEN2*, and *MAPT*) are not encoded on Hsa21, and therefore are not directly relevant to AD-DS.

In studying mouse phenotypes to understand AD-DS, we are presented with two key issues. Firstly, we need to test longitudinally DS models to look for changes in older mice that are not apparent early on, and so may indicate aging or neurodegenerative processes rather than neurodevelopmental deficits. Secondly, we need to separate normal aging processes in DS from those connected specifically to AD-DS. The thoughtful use of the increasing range of different mouse models is enabling us to dissect these issues to further our understanding of AD-DS.

A study that has addressed both (1) neurodegenerative vs. neurodevelopmental and (2) normal aging vs. AD phenotypes has been performed in the Ts65Dn mouse. This study concerned the neurodegenerative phenotype loss of basal forebrain cholinergic neurons (BFCNs), and was carried out through an experimental design involving optimal crossing of different mouse models and assessment of the genetically-distinct progeny (Salehi et al., 2006). Firstly, Salehi and colleagues quantified the known loss of BFCNs in Ts65Dn mice, and showed this loss to be progressive, thus an aging or an AD-related phenotype in this DS mouse model. The authors then compared BFCN loss in Ts65Dn and Ts1Cje DS mouse models (**Figure 1**), and were able to map a dosage-sensitive critical region that had to contain a candidate gene for this phenotype: Ts65Dn mice lose BFCNs but Ts1Cje mice turned out to have no loss compared to wildtype mice. Therefore, the dosage-sensitive gene(s), that when present in three copies is responsible for BFCN loss, must map within the region of trisomy present in Ts65Dn but not in Ts1Cje. A key candidate in this region was the *App* gene. By crossing Ts65Dn mice to heterozygous *App* knockout mice, the authors generated cohorts of progeny that carried the trisomic region with either two or three copies of wildtype *App*. Assessing BFCN loss in these cohorts led to the conclusion that the phenotype arises mainly from having three copies of *App* and, further, that it is associated with impairments in nerve growth factor retrograde transport, linked to early endosomes, which are enlarged (Salehi et al., 2006).

Given the role of *APP* triplication in this phenotype, there is likely a strong link to AD and AD-DS. In people with early AD pathology or mild cognitive impairment, neurofibrillary pathology has been detected in BFCNs (Mesulam et al., 2004; Grudzien et al., 2007), while their loss has been observed in patients with SAD (and other neurodegenerative disorders; Zarow et al., 2003). Interestingly, enlarged early endosomes have been detected in cortical tissues from cognitively intact individuals with mild AD pathology, and in young individuals

with DS (under 12 years old), suggesting that endosome enlargement is an early feature in AD pathogenesis (Cataldo et al., 2000).

DS Models in the Study of Candidate Genes Influencing AD

As illustrated in **Table 1**, while people with DS have three copies of *APP* and develop early AD neuropathology, their clinical presentation is variable, suggesting that other genetic and environmental factors influence pathogenesis. In addition to *APP*, many genes on Hsa21 have been studied in the context of neurodegeneration and/or AD, and it is conceivable that a three-copy dose of any of these genes could contribute to disease and dysfunction.

Single gene overexpressing transgenics do not model DS, or AD-DS, but may provide some insights if carefully considered. For example, seizures and neuronal network abnormalities remain challenging areas to investigate but important phenotypes to be explored in DS, AD-DS, and *APP* overexpression models of AD (i.e., which are single gene transgenic models). In SAD, seizures have been associated with early cognitive decline (Vossel et al., 2013), while the incidence of seizures in AD-DS is high and is associated with increased risk of dementia (for example, McCarron et al., 2014). To date, seizure phenotypes and epileptiform activity have been characterized across numerous *APP* transgenic mice (Born, 2015), but it is unclear whether these phenotypes are primarily driven by amyloid overproduction (Mucke and Selkoe, 2012) or are an effect of unphysiological *APP* overexpression during development (Born et al., 2014). Antiepileptic drugs, such as levetiracetam, which improve seizures in DS (Sangani et al., 2010) and in AD (Cumbo and Ligori, 2010), also ameliorate synaptic and memory dysfunctions in APP transgenic mice by suppressing neuronal network dysfunction (Sanchez et al., 2012; Devi and Ohno, 2013).

So, while single gene transgenic models do not model human trisomy 21 or AD because they usually express the gene by many-fold, from ectopic promoters, they offer insights into some of the functional consequences of overexpression, albeit at non-trisomic levels. **Table 5** presents a list of Hsa21 gene candidates, in chromosomal order, that have been investigated for overexpression-related phenotypes linked with AD across different mouse, fruitfly, and cellular models. We also compare, where data are available, how related changes in these genes have been explored in humans with AD and/or DS. Making optimal use of mouse genetics, some of the single-gene-overexpressing mouse transgenics have been crossed with AD models, to look for changes in phenotypes that may be informative. For example, crossing an *S100β* overexpression model with the Tg2576 *APP* transgenic mouse generates double mutant progeny with exacerbated cerebral amyloidosis and reactive gliosis. This suggests that increased expression of *S100β* could contribute to AD pathogenesis possibly by promoting amyloidogenic APP processing (Mori et al., 2010).

Other key Hsa21 gene candidates *DYRK1A* and *RCAN1* have been linked to AD pathogenesis through their effects on tau. The toxic neurofibrillary tangles (NFTs) that accumulate in AD are formed of hyperphosphorylated tau protein. Overexpression of *DYRK1A* in transgenic mice resulted in tau hyperphosphorylation (Ryoo et al., 2007, 2008), and DYRK1A has been shown to co-localize with NFTs more frequently in AD-DS brain compared to SAD (Wegiel et al., 2008). Similarly, overexpression of *RCAN1* in a mouse model resulted in abnormal tau hyperphosphorylation (Wegiel et al., 2011). This suggests that the increased expression of *DYRK1A* and *RCAN1* in DS could promote the formation of NFTs, a hallmark feature of AD pathology.

Triplication of Hsa21 genes in DS does not necessarily lead to a 1.5-fold increase (compared to euploid individuals) in their RNA or protein expression. For example, a study in DS fetal cortical tissue revealed multiple Hsa21 proteins in fact expressed at similar or lower levels than in disomic controls (Cheon et al., 2003a,b,c,d). Assessments at transcriptomic and proteomic levels, together with meta-analysis across these studies, provide useful resources for understanding patterns of alteration in gene expression (for example, see Vilardell et al., 2011). As a few of the studies in **Table 5** have demonstrated, it is important to verify the effect of trisomy on candidate gene expression, in relevant tissues and contexts, before further characterization of any potential downstream effects of trisomy.

Prospects for Research

Individuals with DS manifest the most common genetic form of AD, and this undoubtedly largely arises from expressing three copies of *APP* (Ness et al., 2012; Hartley et al., 2015). Therefore, studying and modeling this population will assist in understanding the contribution of APP to AD pathogenesis, and evaluating the amyloid cascade hypothesis. However, the variation in clinical presentation of AD-DS shows that many other genetic and environmental factors contribute, almost certainly including protective factors. The thoughtful use of models will thus provide insight into these factors.

To study mouse models of AD-DS, it is critical to dissect neurodevelopmental from neurodegenerative effects (Bothwell and Giniger, 2000; Contestabile et al., 2010). To be of interest for AD-DS, such phenotypes should differ from normal aging in the mouse strain of interest, although this can be difficult to determine, particularly as DS has been characterized as a syndrome of accelerated aging in both clinical (Lott, 2012; Zigman, 2013) and epigenetic terms (Horvath et al., 2015), and because aging remains the clearest non-genetic risk factor for all forms of AD (Fratiglioni, 1996; Bush and Beail, 2004). The longitudinal study of cognitive decline in DS mice poses similar challenges to those in people with DS, and tests need to distinguish between dysfunction due to dementia, as opposed to aging or baseline learning deficits. For example, variations of a learning procedure involving incremental repeated acquisition tasks suggest that declining performances by Ts65Dn mice with age may be due to motor impairments and/or decreased motivation, rather than neurodegenerative-related effects (Sanders et al., 2009). To improve behavioral testing in mouse models of AD-DS, a potential avenue to explore

TABLE 5 | Single gene overexpression models from Hsa21, with relevance to AD phenotypes. Genes are listed in order from centromere to Hsa21q telomere.

Hsa21 gene	Phenotypes studied in models	Phenotypes studied in humans
APP	Please refer to **Table 3**.	Please refer to **Table 1**
SOD1	*SOD1* overexpression protects against APP-induced lethality in transgenic mice (Carlson et al., 1997)	SOD1 activity positively correlates with levels of memory functioning in DS adults (Zis et al., 2012)
ITSN1	Overexpression of *ITSN1* homolog *nla* in combination with *SYNJ1* and *RCAN1* homologs causes impaired vesicle recycling in *Drosophila* (Chang and Min, 2009)	ITSN1 protein (Hunter et al., 2011) and mRNA (Pucharcos et al., 1999) elevated in DS *ITSN1* highly expressed in AD brain (Blalock et al., 2004; Wilmot et al., 2008)
SYNJ1	Mice overexpressing *SYNJ1* have deficits in synaptic transmission (Voronov et al., 2008) *SYNJ1* transgenic mice display enlarged endosomes (Cossec et al., 2012)	SYNJ1 levels higher in DS brain tissue compared to controls, and elevated in AD-DS cases (Martin et al., 2014)
OLIG2	Neural progenitors from *Olig2*-overexpressing mice exhibit impairments in neural progenitor proliferation (Lu et al., 2012)	SNPs in *OLIG2* associated with psychotic symptoms in AD (Sims et al., 2009)
RCAN1	*RCAN1* overexpression in a mouse model causes abnormal tau phosphorylation (Wegiel et al., 2011) In cell models, *RCAN1* overexpression leads to deficits in synaptic transmission (Martin et al., 2012) and promotes neuronal apoptosis (Sun et al., 2011, 2014)	*RCAN1* chronically elevated in AD and DS (Ermak et al., 2001)
DYRK1A	*DYRK1A* overexpression linked to tau hyperphosphorylation and increased Aβ production in transgenic mice (Ryoo et al., 2007, 2008) and cellular models (Park et al., 2007; Coutadeur et al., 2015) *Dyrk1a* overexpression causes phosphorylation of PS1, increasing γ-secretase activity in cells and stabilizing γ-secretase complex in mice (Ryu et al., 2010) Mouse *Dyrk1a* overexpression in TgDyrk1A mice results in a significant reduction of *Rest* mRNA (Canzonetta et al., 2008)	DYRK1A increased in the brains of patients with AD (Kimura et al., 2007) and DS (Ryoo et al., 2008) DYRK1A expression in DS brain correlates with 3-repeat tau levels (Shi et al., 2008; Wegiel et al., 2011) Plasma DYRK1A positively correlates with cerebrospinal fluid tau and phospho-tau in AD patients (Janel et al., 2014) Co-localization of DYRK1A with NFTs greater in AD-DS than SAD (Wegiel et al., 2008) REST levels correlate with cognitive preservation and longevity in aging and are downregulated in AD (Lu et al., 2014)
DSCAM	Trisomy of *Dscam* in *Drosophila* results in synaptic targeting errors (Cvetkovska et al., 2013)	DSCAM overexpressed in a DS patient, and DSCAM immunoreactivity associated with Aβ plaques in demented DS patients (Saito et al., 2000)
ETS2	*Ets2* transgenic mice and fibroblasts overexpressing ETS2 have elevated APP, presenilin1 protein and increased Aβ production (Wolvetang et al., 2003b) *Ets2* overexpression causes apoptosis via caspase 3 activation in primary neuronal cultures (Wolvetang et al., 2003a) and in DS cortical neurons (Helguera et al., 2005)	ETS2 immunoreactivity associated with intracellular Aβ and hyperphosphorylated tau in both AD-DS and sporadic AD brain tissue (Helguera et al., 2005)
BACE2	*BACE2* overexpression *in vitro* reduces Aβ levels (Sun et al., 2006) In a mouse model, overexpression of *BACE2* has no effect on Aβ production (Azkona et al., 2010a,b)	*BACE2* polymorphisms may predict age of onset of dementia in DS (Myllykangas et al., 2005; Mok et al., 2014)
ABCG1	*ABCG1* overexpression stimulates cholesterol efflux *in vitro* (Kim et al., 2007; Tansley et al., 2007) and either reduces (Kim et al., 2007) or increases Aβ production (Tansley et al., 2007), the latter through an increase in APP processing *ABCG1* overexpression in a mouse model has no effect on reference or working memory or synaptic plasticity (Parkinson et al., 2009), nor alters Aβ, APOE nor cholesterol efflux *in vivo* (Burgess et al., 2008)	*ABCG1* gene upregulated in patients with DS (Tansley et al., 2007; Kong et al., 2015) *ABCG1* gene expression unaltered in AD (Tansley et al., 2007)
CSTB	*Cstb* overexpression in a mouse model does not induce epileptic activity or a myoclonic seizure phenotype (Brault et al., 2011)	CSTB protein unaltered in DS fetal cerebral cortex (Cheon et al., 2003b).

(Continued)

TABLE 5 | Continued

Hsa21 gene	Phenotypes studied in models	Phenotypes studied in humans
SUMO3	*SUMO3* overexpression in cell culture systems shown to both increase (Dorval et al., 2007) and reduce (Zhang and Sarge, 2008) Aβ levels *SUMO3* overexpression modulates APP processing, increasing the CTF/APP ratio *in vitro* (Dorval et al., 2007)	High molecular weight SUMO3 conjugates decreased in AD brain tissue (Lee et al., 2014)
S100β	S100β application results in tau hyperphosphorylation in cultured neural stem cells (Esposito et al., 2008) *S100β* overexpression increases neuronal death and reduces neuronal production in DS stem cells (Lu et al., 2011) *S100β* overexpression in Tg2576 AD mice increases Aβ deposition and BACE1 activity (Mori et al., 2010) Mice overexpressing *S100β* show accelerated signs of aging (Shapiro and Whitaker-Azmitia, 2004) neuropathology (Shapiro et al., 2004) and behavioral deficits (Borella et al., 2003)	*S100β* upregulated in DS and AD (Griffin et al., 1989; Sheng et al., 1994) *S100β* overexpression positively correlates with age in DS patients (Royston et al., 1999)

SOD1, superoxide dismutase1; ITSN1, intersectin 1; SYNJ1, synaptojanin 1; OLIG2, oligodendrocyte transcription factor 2; RCAN1, regulator of calcineurin 1; DYRK1A, Dual specificity tyrosine-phosphorylation-regulated kinase 1A; DSCAM, Down syndrome cell adhesion molecule; ETS2, V-Ets Avian Erythroblastosis Virus E26 Oncogene Homolog 2; BACE2, beta-site APP cleaving enzyme 2; ABCG1, ATP-binding cassette sub-family G member 1; CSTB, cystatin B; SUMO3, small ubiquitin-like modifier 3; S100β, S100 calcium binding protein β; REST, repressor element-1 silencing transcription factor.

capitalizes on the association of dementia with deficits in episodic memory. The development of tests based on, for example, visuo-spatial data, should therefore highlight age-dependent, dementia-related deficits in mouse models, because they rely on the encoding and binding of information spontaneously, and do not challenge other cognitive domains (Iordanova et al., 2009).

As well as the hypothesis-driven study of AD-DS phenotypes, one of the greatest strengths of working with mouse models is our ability to undertake unbiased hypothesis-generating research, by mapping phenotypes to genomic critical regions using the range of strains now available. These include chromosome-engineered panels of partially trisomic mice (**Figure 1**) as well as single gene knockout animals, such as the $App^{+/-}$ heterozygous mice, which may be crossed to partially trisomic strains, to generate progeny with altered single gene copy numbers on different trisomic region backgrounds. The cohorts of progeny from these crosses provide ideal groups for testing the contributions of single Hsa21 genes to AD-DS.

Mouse genome engineering continues to offer new models and approaches for teasing apart AD-DS relevant phenotypes, and new strains are being published regularly to help refine experimental strategies. For example, the recent genomically humanized NLF mouse (Saito et al., 2014), which has human amino acid residues at key sites within APP that affect its processing, may yield new insights into the biology of both AD and AD-DS, partly through expressing mutant *APP* at physiological levels. The strategic breeding of new *APP* models with DS segmental trisomies will contribute to determining which phenotypes are downstream of an amyloid cascade. Furthermore, independent study of partial trisomies without three copies of *App* may help tease out effects of other factors, for example oxidative stress, cholesterol metabolism or immune system dysfunction, in the development of dementia (Wiseman et al., 2015).

DS mouse models also give us the flexibility to investigate the effects of potentially dosage-sensitive non-coding regions. For example, microRNAs (miRs)—short (20–23 nucleotide) RNAs that downregulate the transcription of target genes—have increasingly been investigated in AD pathogenesis due to their differential regulation in molecular pathways associated with AD (Veerappan et al., 2013). Hsa21 encodes 29 miRs (MirBase release 21, Griffiths-Jones, 2004), and their potential overexpression in trisomy may contribute to genetic dysregulation relevant to AD-DS. Overexpression of the Hsa21-encoded miR-155 in DS has been reported to increase Aβ production via the downregulation of sorting nexin 27, a membrane-trafficking component found in early endosomes, that modulates γ-secretase activity (Wang et al., 2013, 2014).

Hsa21 also encodes genes involved in post-translational histone modification, including *DYRK1A*, *ETS2*, *HMGN1*, *BRWD1,* and *RUNX1* (Dekker et al., 2014), which may be investigated for their potential roles leading to the aberrant histone modifications observed in AD (Zhang et al., 2012a; Narayan et al., 2015). Histone methylation (specifically H3K4me3) has been shown to correlate highly with genome-wide domains of dysregulated gene expression in DS, which are highly conserved between humans and Ts65Dn mice (Letourneau et al., 2014). DS mouse models therefore model epigenetic structures in humans and may be used to study the effects of its dysregulation in AD-DS.

Finally, mouse model research must be undertaken in parallel with other rapid advances in the AD-DS field. The advent of human induced pluripotent stem (iPS) cells (Hunsberger et al., 2015) for DS provides for the first time a trisomic human *in vitro* model that recapitulates hallmarks of some AD pathology (Shi et al., 2012; Chang et al., 2015; Moore et al., 2015; Murray et al., 2015). The further development of this technology (Hunsberger et al., 2015) will prove valuable to phenotyping and drug target discovery, alongside *in vivo* research

and *in vitro* primary cultures from DS mice. An increasing call is being made for partnerships to build up large cohorts of, and biobanks from, people with DS for the systematic longitudinal study of AD-DS progression (Hartley et al., 2015). In-depth phenotypic studies across development with infants and adults with DS are already underway (Wiseman et al., 2015). These will allow greater power to identify biomarkers for the prediction of AD in this large, genetically well-defined population, for example, through plasma (Dekker et al., 2015; Schupf et al., 2015), cerebrospinal fluid (Portelius et al., 2014a,b), and neuroimaging studies (Beacher et al., 2009; Landt et al., 2011; Powell et al., 2014; Sabbagh et al., 2015). Biomarker studies are also being performed in AD models, including at very early phases of Aβ deposition (Maia et al., 2015). Extending these studies to mouse models of DS and AD-DS will contribute to elucidating the genotype-phenotype relationships that ultimately lead to dementia.

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REVIEW
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Pain perception in people with Down syndrome: a synthesis of clinical and experimental research

Brian E. McGuire[1]* *and Ruth Defrin*[2]

[1] School of Psychology and Centre for Pain Research, National University of Ireland, Galway, Ireland, [2] Department of Physical Therapy at the Sackler Faculty of Medicine and Sagol School of Neuroscience, Tel-Aviv University, Tel-Aviv, Israel

People with an intellectual disability experience both acute and chronic pain with at least the same frequency as the general population. However, considerably less is known about the pain perception of people with Down syndrome. In this review paper, we evaluated the available clinical and experimental evidence. Some experimental studies of acute pain have indicated that pain threshold was higher than normal but only when using a reaction time method to measure pain sensitivity. However, when reaction time is not part of the calculation of the pain threshold, pain sensitivity in people with Down syndrome is in fact lower than normal (more sensitive to pain). Clinical studies of chronic pain have shown that people with an intellectual disability experience chronic pain and within that population, people with Down syndrome also experience chronic pain, but the precise prevalence of chronic pain in Down syndrome has yet to be established. Taken together, the literature suggests that people with Down syndrome experience pain, both acute and chronic, with at least the same frequency as the rest of the population. Furthermore, the evidence suggests that although acute pain expression appears to be delayed, once pain is registered, there appears to be a magnified pain response. We conclude by proposing an agenda for future research in this area.

Keywords: pain, Down syndrome, intellectual disability

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George T. Capone, Kennedy Krieger Institute, USA

***Correspondence:**
Brian E. McGuire, School of Psychology and Centre for Pain Research, National University of Ireland, University Road, Galway, Ireland
brian.mcguire@nuigalway.ie

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Pain in Intellectual Disability and Down Syndrome

Until recently, the commonly held view was that individuals with intellectual disability have decreased sensitivity to pain (e.g., Biersdorff, 1994; Feldt et al., 1998). This view was based on a number of factors such as the tendency of people with an intellectual disability not to report pain in potentially harmful situations and from observations of high rates of self-injurious behavior amongst some individuals with intellectual disability. However, recent reviews have cast doubt on these assumptions and have pointed instead to difficulties identifying pain in people with impaired (or different) means of communication and the presence of behavioral expressions of pain that may be variable and idiosyncratic (McGuire and Kennedy, 2013). Furthermore, the conceptual term "intellectual disability" captures a wide range of conditions, most of which are not well characterized and which are often of unknown etiology, making any generalization quite challenging. Notwithstanding these caveats, there are a number of reasons to suspect that people with intellectual disability may be at increased risk for experiencing chronic pain, including a possible heightened sensitivity to pain (Defrin et al., 2004), low levels of physical activity (Robertson et al., 2000), increased risk of accidental injury

(Sherrard et al., 2001), reduced involvement in health decision making (McGuire et al., 2007), more physical co-morbidities (Baldridge and Andrasik, 2010) and reduced use of services for management of pain (McGuire et al., 2010).

While an intellectual disability is typically part of the clinical picture of Down syndrome, more is known about the specific features of Down syndrome than is the case when talking about intellectual disability as a more generic construct. People with Down syndrome may have specific risks for experiencing chronic pain because of increased risk of potentially painful conditions. These are synthesized here with approximate prevalence vales: congenital heart anomalies (15%), acquired cardiac disease (16%), chronic pulmonary changes (30%), osteoarthritic degeneration of the spine (32%), osteoporosis with resultant fractures of the long bones (55%) or vertebral bodies (30%), untreated atlanto-occipital instability (8%), eye problems (36%), celiac disease (11%), eczema (23%) (see for example; van Allen et al., 1999; Henderson et al., 2007; Hansdorfer et al., 2013).

For the purpose of this review we searched computerized databases (Pubmed, Medline, Scopus and Web of Science), published bibliographies of related topics, and references provided by colleagues. We limited our review to publications in the years 1960–2014. For this brief review, we have not attempted to conduct an exhaustive systematic review, but instead have selected literature germane to focus of our paper. Wherever possible in this paper, we will focus on the evidence regarding Down syndrome specifically, although in many studies the population described is a more general intellectual disability group of which some have Down syndrome. We will make the distinction wherever the literature allows us to.

Pain Sensitivity in Down Syndrome

In order to determine whether pain sensitivity is altered in people with Down syndrome, it is necessary to have methods by which pain sensitivity can be measured accurately. Sensitivity to pain is measured experimentally by introducing subjects to stimuli of ascending or descending order until the boundary of pain is reached, a procedure termed "method of limits". Another psychophysical method is the procedure of repeatedly introducing stimuli of various intensities and determining the threshold within the range of the stimulus that can almost never be detected, and that which is almost always detected as painful ("method of constant stimuli"). In both methods, pain threshold is defined as the smallest amount of stimulus energy (or intensity) necessary to evoke pain (Gescheider, 1985). The responses of the subjects to the stimuli in either method is based on self-report by way of verbal expression, body language, or withdrawal of the affected body part from the painful stimulus.

Only a few studies have actually measured pain threshold in individuals with Down syndrome. In the first study of its kind, Hennequin et al. (2000) evaluated pain threshold among 9 children and 17 adults with Down syndrome (age range 4–30 years) by measuring the time elapsed from the application of an ice cube on their wrist and temple to the first verbal expression of pain. The onset of verbal response was longer in individuals with Down syndrome compared with controls, suggesting a higher pain threshold (less sensitivity to pain) in the former. Valkenburg et al. (2015) has measured cold- and heat-pain thresholds, using computerized thermal stimulator among 21 children with Down syndrome (ages 10–15 years). The study measured the thresholds with the reaction-time dependent method of limits wherein subjects (or examiners) are required to press a switch the moment they perceive pain, thus ceasing the increase in stimulation intensity. Similar to Hennequin et al. (2000), the authors found higher thresholds compared to the participants' siblings. However, in both of these studies, the pain threshold was affected by the reaction time of the individual and by the conduction velocity of the nervous system.

In an attempt to evaluate the effect of the pain measurement method on individual responses, Defrin et al. (2004) measured heat-pain threshold with two different methods; a reaction-time dependent method (method of limits) and a reaction-time free method (method of levels). In contrast to the method of limits, stimuli in the method of levels are predetermined and therefore their intensity is not affected by the subject's performance nor by the conductance of the nervous system. Using a computerized thermal stimulator, 25 adults with an intellectual disability were tested, 14 of whom had Down syndrome (ages 22–56 years). When tested with the method of limits, individuals with Down syndrome exhibited pain threshold similar to that of age- and sex-matched cognitively intact controls. However, when tested with the method of levels, a significant group effect emerged wherein individuals with Down syndrome had a significantly lower pain threshold than controls (i.e., more sensitive to pain). These results suggested that individuals with Down syndrome are more sensitive to pain than normal but that slower reaction time gives the impression that pain threshold is higher than it actually is. Thus, this impression of reduced pain sensitivity appears to be an artifact associated with the method of measuring pain sensitivity and when reaction time is controlled for, there is evidence that people with Down syndrome are more sensitive to pain than average.

Reaction-Time in Down Syndrome and its Relation to Pain Sensitivity

In evaluating the literature on pain sensitivity in Down syndrome, a question arises about the possible confound of: (a) capacity to report pain quickly; (b) possible altered somatosensory processes in Down syndrome. Some studies suggest that median nerve conduction velocities, (i.e., the speed at which action potentials evoked by electrical stimulation propagate along the median nerve) but not scalp-evoked potential latencies, were slower and their amplitude lower among children and adults with Down syndrome when compared with controls. This suggests impaired peripheral somatosensory function in Down syndrome (Kakigi, 1989; Brandt and Rosén, 1995).

In contrast, normal peripheral conduction velocity but prolonged latencies of somatosensory evoked potentials were recorded among infants or young adults with Down syndrome (Straumanis et al., 1973; Chen and Fang, 2005), suggesting that

central conduction and/or processing of the nociceptive signals is delayed. The inter-hemispheric transmission time of adults with Down syndrome was also longer than in controls (Heath et al., 2007), further pointing to delays in central processing. Although these studies used innocuous stimuli, it is possible that conduction and processing of noxious stimuli may also be impaired and underlie the apparent decreased pain sensitivity when methods of sensitivity depend on conduction. Indeed, indirect evaluations of conduction velocity and reaction time based on responses to noxious thermal stimuli reveal slower times compared to controls in individuals with Down syndrome (Defrin et al., 2004; Valkenburg et al., 2011) and also compared to individuals with unspecified intellectual disability (Defrin et al., 2004). This may suggest a specific (slower) pain response in Down syndrome that is not attributable simply to the presence of intellectual disability. Consequently, measurements of pain that depend on reaction time or conduction velocity may portray a misleading hyposensitivity to pain due to delayed responses.

Verbal Reports of Pain Among Individuals with Down Syndrome

Interviewing individuals with intellectual disability is important because, depending on their level of cognitive impairment, they can be the best source of information regarding their health. Despite this, people with intellectual disability are rarely involved in making important decisions regarding their health (McGuire et al., 2007). Little is known about the ability of individuals with Down syndrome to provide an adequate self-report of pain. This was evaluated in only a few studies. Zabalia and Corfec (2008) asked children and adolescents with Down syndrome to assess the pain of characters in pictures, using a FACES rating scale depicting pain responses and a visual-analog scale (a Likert-type scale). The children with Down syndrome were able to identify emotions and pain similar to children without Down syndrome, especially when using the FACES scale. de Knegt et al. (2013) introduced two rating scales; FACES and a numerical rating scale, to 106 adults with Down syndrome. The authors found that although participants better understood the FACES scale, 70% comprehended at least one of the two scales.

As part of a European initiative on pain in cognitive impairment (European Cooperation in the Field of Scientific and Technical Research [COST], 2015, TD-1005), our group has recently conducted an experimental study in which 29 adults with mild-moderate intellectual disability received pressure stimuli of various intensities during which time pain ratings were obtained using a pyramid pain rating scale. The pyramid scale is constructed of five pyramids of increasing sizes and heights expressing different pain intensities. The pain ratings of the nine participants with Down syndrome (age 31–36) correlated significantly with pressure intensity, suggesting that they could provide adequate ratings of their pain using this (graphical) scale (Benromano et al., 2015).

Results concerning pain self-report abilities of individuals with intellectual disability other than Down syndrome are inconsistent (e.g., Dagnan and Ruddick, 1995; McGrath et al., 1996; LaChapelle et al., 1999; Chibnall and Tait, 2001; Defrin et al., 2006). Generally, the reliability of self-report is usually inversely correlated with the intellectual disability level, with those less affected being more reliable in their ratings. Furthermore, graphical or 3 dimensional scales (e.g., the Poker Chip Tool) might be more suitable than two-dimensional verbal or numeric scales. Further study is needed to explore the best rating scale(s) for individuals with Down syndrome. In any case, the inability to report pain does not mean pain is not present (International Association for the Study of Pain, 1994) and the inability to use formal pain rating scales does not preclude the ability to provide free verbal report of the existence of pain, even as a gross indicator of the presence of pain.

Behavioral Indicators of Pain

As not all individuals with intellectual disability can verbally communicate their pain, other indirect methods have been used to assess pain perception based on observation of manifestations that are considered indicators of pain. In very young children, intelligible verbal report of pain has not yet developed but basic vocalizations of pain may be present (such as crying) and there may be facial indicators or physiological indicators (such as heart rate or respiration rate).

Although frequently used in studies of general intellectual disability (LaChapelle et al., 1999; Nader et al., 2004; Dubois et al., 2010; Rattaz et al., 2013), only a few studies have examined behavioral or physiological indicators of pain specifically in Down syndrome. In an early study, Lind et al. (1970) found that babies with Down syndrome required more stimulation to provoke crying and had diminished visible responses to pain than control babies. More recently, Valkenburg et al. (2011) used the COMFORT-B scale (that includes manifestations such as alertness, respiratory response, body movements and crying) to assess post-operative pain among 76 new born babies and children with Down syndrome. Although mean COMFORT-B scores of children with Down syndrome were higher than comparable controls, the scores did not differ significantly between the groups. Aguilar Cordero et al. (2015) found that behavioral (e.g., crying) and physiological responses (oxygen saturation, heart rate, blood pressure) following vein/heel puncture were slower among 20 new born babies with Down syndrome and not as clearly defined as that of babies without Down syndrome. However, when pain was finally perceived, it persisted for a longer time among infants with Down syndrome. As with sensory testing, it appears that physiological manifestations of pain in new born babies with Down syndrome emerge more slowly than normal, but once registered, they may represent enhanced or prolonged pain experience. This may suggest either a magnified nociceptive process or a delayed or inefficient inhibitory response.

Facial expressions of pain following pressure stimuli among individuals with intellectual disability have recently been analyzed by our group in the aforementioned experimental study (Benromano et al., 2015), using the Facial Action Coding System (FACS; Ekman and Friesen, 1978). The nine participants with Down syndrome had significantly increased facial expressions compared to cognitively intact controls, both at baseline and

throughout stimulation intensities. FACS scores correlated with stimulation intensity, suggesting that facial expressions can reliably indicate the intensity of pain among individuals with Down syndrome (Benromano et al., 2015). However, as there are also increased facial expressions at rest that could be interpreted as pain expression, additional measures of pain are advised. Kyrkou (2005) noted that a pale face and restlessness indicated the presence of menstrual pain among women with Down syndrome. We could not find additional studies that focused on facial/bodily expressions of pain specifically in Down syndrome. Nevertheless, several studies included people with Down syndrome among the tested populations and reported that as a whole, individuals with intellectual disability exhibit significant elevations in facial expressions/bodily movements during painful events as compared to baseline (e.g., Breau et al., 2002; Benini et al., 2004; Defrin et al., 2006; Dubois et al., 2010).

Some authors noted that individuals who were unable to verbalize their pain tended to exhibit atypical pain expressions such as freezing, smiling and hand flapping/rubbing (Defrin et al., 2006; Dubois et al., 2010). Such expressions, which are unexpected in the context of pain, may mislead observers to think that the individual is not in pain. Thus, measurements of behavioral indicators of pain might prove useful in quantifying pain among individuals with Down syndrome, but the optimal scale for doing so has yet to be determined.

Caregiver Evaluation of Pain

A number of studies have attempted to estimate the extent of pain in people with general intellectual disability based on caregiver report. For example, two recent studies estimated that 13–15% of people with intellectual disability have chronic pain based on caregiver report (McGuire et al., 2010; Walsh et al., 2011), but concluded that pain was likely to be under-recognized and under-treated as a result (McGuire et al., 2010). This conclusion was based on the fact that third party evaluation of pain is very challenging, even for parents. For example, around 30% of parents of children with Down syndrome had difficulty perceiving if their child was in pain and 70% of the parents had difficulty identifying the location of the pain (Hennequin et al., 2003). The likelihood of parents reporting difficulty in discerning if and where their child with Down syndrome had pain was *greater* than for a sibling without Down syndrome. It is noteworthy, however, that parents of children with intact cognition may also experience difficulties in identifying the amount of pain experienced by their children (e.g., Jylli and Olsson, 1995; Chambers et al., 1998; Larochette et al., 2006). Thus, while parents are more familiar with their child's typical pain reactions than other care takers, parents may still underestimate and overestimate pain, even in verbal children.

Davies (2010) recently reported that parents of children with Down syndrome assessed their child's pain through the child's verbalizations (words, showing pain location and crying), behavioral expressions (changes in usual activities, seeking closeness to the parent) and emotional changes (e.g., anger, fear, frustration and acting out). The parents reported that they also assessed pain based on their beliefs that the child was less verbal, slower to complain, and less bothered by pain than siblings. In another study, parents reported in 66% of the cases that their child was less sensitive to pain than normal, although there is some evidence that children with Down syndrome are more sensitive to heat/cold pain (Valkenburg, 2012). While knowledge of the idiosyncratic behaviors of their children will facilitate parents in recognising pain in their child with Down syndrome, this is more challenging for other caregivers such as teachers or health professionals. The unique pain expression of some people with Down syndrome may mislead caregivers.

Imaging Studies in Down Syndrome and Implications for Pain Perception

Magnetic resonance imaging (MRI) studies reveal distinctive alterations in brain anatomy among individuals with Down syndrome. For example, there is evidence of smaller overall brain volume, disproportionately smaller cerebellar volume and relatively larger subcortical gray matter volume in people with Down syndrome compared to controls (Pinter et al., 2001). Aging occurs prematurely in Down syndrome and manifests in neuropathological atrophies typical of Alzheimer's disease including, but not restricted to, reduction in hippocampal, parietal, orbitofrontal, lingual and post central volume (Teipel et al., 2004; Teipel and Hampel, 2006; Koran et al., 2014). The effects of these neuropathological changes on pain perception and behavioral expression of pain in Down syndrome is not known.

Functional connectivity MRI studies reveal higher regional connectivity in the ventral brain system (the amygdala and anterior temporal region and the ventral aspect of the anterior cingulate and frontal cortices) and lower connectivity in dorsal executive networks (dorsal prefrontal, anterior cingulate and posterior insula cortices; Pujol et al., 2015). These changes may affect the experience of pain. For example, the orbitofrontal cortex is involved in pain modulation via brain stem structures (Lorenz et al., 2003; Zeidan et al., 2011); reduced volume and connectivity of which may reduce pain modulation and thus increase the intensity of perceived pain among individuals with Down syndrome. Furthermore, structural, and related functional alterations in the insular and cingular as well as somatosensory cortices may induce alterations in processing of the sensory and affective aspects of pain (Davis and Moayedi, 2013). The connectivity increases and decreases found in Down syndrome are thought to account for reduced adaptive behavior, which in turn is related to communication skills (Pujol et al., 2015) and may thus also account for delayed and altered behavioral responses to pain, as described above.

Modifying Pain Experience in People with Down Syndrome

Current models of pain conceptualize pain perception as being the consequence of integrating several sources of information including sensory information, cognitive appraisals of the pain, emotional responses, behavioral responses and social context. Thus far, both experimental and clinical studies of pain perception in people with Down syndrome have tended to

focus on the sensory component of pain. Yet, in the broader population, many studies have evaluated methods for assisting with modifying both acute and chronic pain experience. For example, attention diversion is a well-established method of coping with pain (e.g., Van Damme et al., 2010) as is cognitive behavioral therapy (CBT; e.g., Eccleston et al., 2012) whereby cognitions and behaviors are modified in order to enhance pain coping. However, virtually no studies have evaluated these methods in people with Down syndrome or intellectual disability more generally, despite the fact that modified CBT has been shown to be effective for treating people with intellectual disability with depression, anxiety and anger problems (McGuire and Kennedy, 2013).

A few notable exceptions have looked at these methods for managing pain in people with an intellectual disability (although not Down syndrome *per se*). For example, a case report study of a person with a mild intellectual disability who had chronic pain indicated that psychological treatments may be of benefit (Lewis et al., 2007). A significant development has been the production of a CBT-based treatment manual ("*Feeling Better*") designed to be used by caregivers to assist people with intellectual disability in developing pain self-management strategies (McManus and McGuire, 2010). In a case series, the authors of the treatment manual reported some preliminary evidence of the effectiveness of the programme (McManus and McGuire, 2014) but noted that more research is needed, including controlled clinical trials. Subsequently, a trial protocol has been registered (Kennedy et al., 2014) to evaluate the effectiveness of the *Feeling Better* programme for management of menstrual pain in young women with an intellectual disability. Treatment outcomes have not yet been reported but will be important as the first controlled trial to evaluate psychological management of pain for people with an intellectual disability.

Animal Models

Studies on animal models of Down syndrome (e.g., the Ts65Dn and APP-SOD1 mice) also indicate delayed response to noxious stimuli compared to control mice (Martínez-Cué et al., 1999; Kotulska et al., 2011). At the same time, there is evidence of increased tissue pathology after induced damage in transgenic animals compared to controls, evident by more prominent neuroma formation, decreased motor neuron survival and impaired regeneration capacity (Kotulska et al., 2011). These studies imply that while Down syndrome is associated with slower conduction of noxious stimuli which may affect the animal's pain behavior, the development of pathology following tissue damage is not delayed and may even be enhanced.

In another study, the response of Ts65Dn mice to neurotrophic factors such as nerve growth factor (NGF) was abnormal (Seo and Isacson, 2005). Although the consequence of this finding is not clear yet, neurotrophic factors were found to promote neuroma formation and enhance pain sensation, potentially underlying the changes found in individual pain thresholds. Further animal studies are needed to determine whether and which alterations exist in the conduction of noxious stimuli in Down syndrome.

Conclusions and Recommendations for Future Studies

On the basis of the limited data on sensory and behavioral testing, we tentatively conclude that individuals with Down syndrome are more sensitive to pain than normal. The evidence suggests that although pain expression appears to be delayed, once pain is registered, there appears to be a magnified pain response. This conclusion corresponds with imaging studies showing differences in structures involved in pain modulation (e.g., frontal cortex) as well as structures involved in pain processing (e.g., cingulate, insula and sensory cortex). Still, some inconsistency exists in pain threshold measurements that may reflect interruption in peripheral conduction and central processing of sensory signals, especially if pain threshold is measured with methods that include reaction time. While such alterations have been reported for innocuous stimuli, studies are needed to prove that such alterations indeed occur in nociceptive pathways. Due to the possibility of delayed reaction time, measuring pain threshold with methods that bypass this limitation, i.e., reaction-time free methods, is preferable. However, pain threshold measurement is suitable only for individuals with mild and perhaps moderate cognitive impairment. Thus, the use of indirect indices of pain is necessary. Additional studies are needed in order to explore which indices best reflect pain in Down syndrome.

While individuals with Down syndrome are at increased risk to experience pain due to congenital and acquired abnormalities and environmental risk factors (e.g., higher risk of accidental injury), they typically have difficulty in expressing their pain and their caregivers face great challenges in identifying and quantifying pain. Thus, from a clinical point of view, it is imperative to investigate pain processing and pain expression of individuals with Down syndrome in both the experimental and clinical setting. Until optimal tools are available for this purpose, caregivers should take into consideration unexpected and sometimes seemingly ambiguous responses to painful incidents. We have previously advocated the use of more than one source of information to identify pain, in order to increase the reliability of the information obtained (McGuire and Kennedy, 2013).

Finally, people with Down syndrome exhibit evidence of premature aging and a greatly increased risk of developing Alzheimer disease (Zigman and Lott, 2007). In the general population, chronic pain is known to affect some 30–50% of people with Alzheimer disease (e.g., Shega et al., 2004; Zwakhalen et al., 2009), and within that population there are enormous challenges in identifying the presence of pain (Corbett et al., 2014) so as to implement an appropriate plan for pain management. No studies have yet examined the problem of pain in people with Down syndrome who also have evidence of onset of Alzheimer-related dementia. This "double jeopardy" represents a major challenge for both researchers and clinicians, but is an important area for future research.

In concluding, we propose the following agenda for future research in the area:

In the clinical domain:

1. More epidemiological studies on the prevalence and profile of chronic pain in people with Down syndrome.
2. Further evaluation of observer-based and self-report pain assessment tools.
3. Better understanding of potentially different pain expression based on the type of pain (e.g., neuropathic, inflammatory) or derivation of pain (e.g., post surgical (for example, tonsils, hip replacement), gastrointestinal, dental etc.).
4. The perception of pain associated with self-injury in low functioning persons with Down syndrome.
5. Evaluation of how the presence of dementia affects the manifestation of pain in people with Down syndrome.
6. A greater emphasis on evaluating pain management interventions, including self-management (psychological coping strategies).

In the experimental domain:

1. Further evaluation of observer-based and self-report pain assessment tools using calibrated noxious stimuli of varying intensities.
2. Measuring physiological and electrophysiological reactions to experimental pain that may potentially replace self report, including but not restricted to heart-rate variability and electromyography (EMG) and electroencephalogram (EEG).
3. Measuring conduction velocity and reaction time to noxious stimuli.
4. Studying pain perception using event related potentials (ERP) and functional magnetic resonance imaging (fMRI) that enable the association between noxious stimulation and activation in specific brain regions of interest.

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frontiers in Behavioral Neuroscience

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Pharmacological correction of excitation/inhibition imbalance in Down syndrome mouse models

*Benoit Souchet[1], Fayçal Guedj[1,2], Zsuza Penke-Verdier[3], Fabrice Daubigney[1], Arnaud Duchon[4], Yann Herault[4], Jean-Charles Bizot[5], Nathalie Janel[1], Nicole Créau[1], Benoit Delatour[3] and Jean M. Delabar[1,3]**

[1] Université Paris Diderot, Sorbonne Paris Cité, Adaptive Functional Biology, UMR Centre National de la Recherche Scientifique 8251, Paris, France, [2] Tufts Medical Center, Mother Infant Research Institute, Boston, MA, USA, [3] Université Pierre-et-Marie-Curie Paris, 06 UMR S 1127, Centre National de la Recherche Scientifique UMR 7225, Institut National de la Santé et de la Recherche Médicale, U 1127, Sorbonne Universités, Institut du Cerveau et de la Moelle Epiniere, Paris, France, [4] Institut Génétique Biologie Moléculaire Cellulaire, Centre National de la Recherche Scientifique, Institut National de la Santé et de la Recherche Médicale, UMR7104, UMR964, Illkirch, France, [5] Key-Obs, Orléans, France

Cognitive impairment in Down syndrome (DS) has been linked to increased synaptic inhibition. The underlying mechanisms remain unknown, but memory deficits are rescued in DS mouse models by drugs targeting GABA receptors. Similarly, administration of epigallocatechin gallate (EGCG)-containing extracts rescues cognitive phenotypes in Ts65Dn mice, potentially through GABA pathway. Some developmental and cognitive alterations have been traced to increased expression of the serine-threonine kinase *DYRK1A* on Hsa21. To better understand excitation/inhibition balance in DS, we investigated the consequences of long-term (1-month) treatment with EGCG-containing extracts in adult mBACtgDyrk1a mice that overexpress *Dyrk1a*. Administration of POL60 rescued components of GABAergic and glutamatergic pathways in cortex and hippocampus but not cerebellum. An intermediate dose (60 mg/kg) of decaffeinated green tea extract (MGTE) acted on components of both GABAergic and glutamatergic pathways and rescued behavioral deficits as demonstrated on the alternating paradigm, but did not rescue protein level of GABA-synthesizing GAD67. These results indicate that excessive synaptic inhibition in people with DS may be attributable, in large part, to increased *DYRK1A* dosage. Thus, controlling the level of active DYRK1A is a clear issue for DS therapy. This study also defines a panel of synaptic markers for further characterization of DS treatments in murine models.

Keywords: Down syndrome, DYRK1A, EGCG, GABA pathway, glutamate pathway, excitation/inhibition balance

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****Correspondence:***
Jean M. Delabar
jeanmaurice.delabar@icm-institute.org

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Introduction

Down syndrome (DS), occurring in 1 in every 750 live births, encompasses a constellation of features caused by partial or complete trisomy for chromosome 21 (Hsa21). In particular, an altered copy number for segments of Hsa21 containing the dual-specificity tyrosine phosphorylated and regulated kinase 1A (*DYRK1a*) gene can induce morphological defects and cognitive impairments (Delabar et al., 1993; Ronan et al., 2007; van Bon et al., 2011). These defects have been reproduced in a number of different mouse models of DS (Ts1Rhr, Ts65Dn, Ts1Cje, Dp(16)1Yey) as

well as mice with altered copy numbers of *Dyrk1a* (hBACtgDyrk1a, hYACtgDyrk1a, mBACtgDyrk1a, *Dyrk1a*+/−). Interestingly, a phenotype rescue experiment crossing Ts65Dn mice, which have three copies of *Dyrk1a*, with mice monosomic for a 33-gene chromosomal segment containing *Dyrk1a* (Ms1Rhr) produced progeny with a normal learning phenotype, indicating that triplication of this 33-gene region is necessary to produce the cognitive deficit (Belichenko et al., 2009). A complete phenotypic assessment of Ts1Rhr mice, trisomic for the 33-gene segment, showed that trisomy of this region is sufficient to produce significant alterations in behavioral tasks such as the open-field, novel object recognition, and T-maze tasks. In Ts65Dn, Ts1Cje, and Ts1Rhr mice, long-term potentiation (LTP) in fascia dentata (FD) could be induced only after blocking GABA(A)-dependent inhibitory neurotransmission. In addition, widespread enlargement of dendritic spines and decreased density of spines in FD were preserved (Haas et al., 2013). Thus, cognitive impairment in DS appears to derive from molecular and structural changes related to an altered copy number within this 33-gene region.

Among the genes from this 33-gene region, *Dyrk1a* is an attractive candidate for inducing cognitive impairment phenotypes. *DYRK1A*, the mammalian ortholog of Drosophila minibrain kinase (mnb) (Tejedor et al., 1995), encodes a proline/arginine-directed serine/threonine kinase. Both in trisomic mice and in individuals with DS, brain levels of DYRK1A are increased approximately 1.5-fold, indicating that this protein is overexpressed in a gene dosage-dependent manner (Dowjat et al., 2007). Further, comparisons of mouse models having different copy numbers of *Dyrk1a* have provided important support for the hypothesized contribution of DYRK1A to cognition. We previously assessed the molecular (i.e., immunoblotting/immunohistochemistry) and behavioral (e.g., rotarod, Morris water maze, Y-maze) consequences of alterations in *Dyrk1a* dosage in mBACtgDyrk1a, Ts65Dn, Dp(16)1Yey (each with 3 gene copies), and *Dyrk1a*+/− (one functional copy) mice (Souchet et al., 2014). Increased expression of DYRK1A in mBACtgDyrk1a induced molecular alterations in synaptic plasticity pathways, particularly expression changes in GABAergic- and glutaminergic-related proteins (Souchet et al., 2014). Similar alterations were observed in models with partial trisomy of Mmu16, Ts65Dn and Dp(16)1Yey, and were reversed in the *Dyrk1a*+/− model. Further, *Dyrk1a* overexpression produced an increased number (using stereological methodology) and an increased signal intensity of neurons expressing GAD67, an enzyme that synthesizes GABA, indicating inhibition pathway alterations in three different models. Functionally, DYRK1A overexpression protected mice from PTZ-induced seizures related to GABAergic neuron plasticity. DYRK1A dosage affects pathways involved in synaptogenesis and synaptic plasticity and influences a shift in E/I balance toward inhibition. Inhibition of DYRK1A activity offers a therapeutic target for DS, but its inhibition/activation may also be relevant for psychiatric disorders with E/I balance alterations.

Many competitive inhibitors targeting the ATP binding site of DYRK1A have been described; most also inhibit secondary targets (Ogawa et al., 2010). A comparative analysis indicates that epigallocatechin gallate (EGCG), a flavanol present in green tea, appears to inhibit DYRK1A with PRAK, another serine/threonine kinase, as a secondary target (Bain et al., 2003). Interestingly, EGCG acts non-competitively at a site external to the ATP binding site (Adayev et al., 2006). We previously assessed the effect of lifelong EGCG treatment, beginning prenatally, on the phenotype of the hYACtgDyrk1a mouse model. A dose of 50 mg/kg resulted in normal memory as measured on the novel object paradigm (Guedj et al., 2009). Following our report, a pilot clinical study performed on a group of young adults with DS found that a decaffeinated green tea extract (Mega Green Tea Extract, MGTE) improved "episodic memory test" results of the patients (De la Torre et al., 2014).

However, the mechanistic basis of the effects of EGCG treatment is not clearly established at the molecular level. Therefore, in the current study we investigated the molecular effects of a commercial green tea extract, POL60, on murine mBACtgDyrk1a and Ts65Dn models, at a dose similar to the one used in our prior report. Specifically, we assessed the effects of treatment on GABA (GAD67, GAD65, VGAT) and glutamate (GLUR1, GLUR2, NR1, NR2A, VGLUT1) pathways and on short-term memory. We also studied the effects of decaffeinated MGTE, used in the pilot clinical trial, and compared them with the effects of POL60 treatment as well as with a caffeine treatment potentially interfering with the effect of EGCG. The findings of these studies offer insights applicable to potential interventions to improve E/I balance in people with DS as well as some psychiatric disorders.

Materials and Methods

Experimental Mice

Mice were housed in standard cages with access to food and water *ad libitum*, under a controlled environment (temperature = 20 ± 1°C; humidity = 60%), and with a light/dark cycle of 12 h. All experiments were conducted in accordance with the ethical standards of French and European regulations (European Communities Council Directive, 86/609/EEC). Official authorization from the French Ministry of Agriculture was granted to perform research and experiments on animals (authorization number 75–369), and the study was approved by the local ethical committee (Univ Paris-Diderot). Mice were fed a standard laboratory diet (CRM, Special Diets Services, Dietex, France Usine). Number of mice and suffering were minimized as possible. Ts65Dn mice (Davisson et al., 1993) were maintained on a B6/C3H background and genotyped as described previously (Reinholdt et al., 2011). Mice carrying the murine BAC containing one copy of *Dyrk1A* (mBACtgDyrk1a) were maintained on a C57BL/6J background and genotyped as described (Guedj et al., 2012). (See Supplementary Table 1).

EGCG Treatment

For EGCG treatment, a final concentration of 225 mg/kg/day of Polyphenon 60 (POL60, Sigma) in water was delivered via drinking water to adult (3–4 months) male mice for 4 weeks for mBACtgDyrk1a, or for 4 weeks before and during behavioral

TABLE 1 | Protein levels of markers of inhibition and excitation pathways for WT and mBACtgDyrk1a (TG) in cortex, hippocampus, and cerebellum following treatment with EGCG-containing POL60 extract.

Inhibitors Comparison Markers	POL 60 -EGCG 67.5 mg/kg			POL 60 -EGCG 67.5 mg/kg			POL 60 -EGCG 67.5 mg/kg		
	TG/WT	TG*/WT	TG*/TG	TG/WT	TG*/WT	TG*/TG	TG/WT	TG*/WT	TG*/TG
	CTX			HPC			CRB		
DYRK1A	162.2 ± 3.3 $p < 0.0001$	174.3 ± 5.4 $p < 0.0001$	174.3 ± 5.4 $p = 0.07$	199.8 ± 9.2 $p < 0.0001$	208.8 ± 17 $p < 0.0001$	208.8 ± 17 $p = 0.64$	208.7 ± 18.4 $p < 0.0001$	145.2 ± 11.8 $p = 0.004$	145.2 ± 11.8 $p = 0.01$
GAD67	131.9 ± 6.6 $p < 0.0006$	108.1 ± 7.1 $p = 0.35$	108.1 ± 7.1 $p = 0.02$	142.2 ± 5.4 $p = 0.01$	109.8 ± 8.4 $p = 0.38$	109.8 ± 8.4 $p = 0.004$	155.8 ± 8.9 $p = 0.003$	117.6 ± 10.8 $p = 0.4$	117.6 ± 10.8 $p = 0.01$
GAD65	136.4 ± 4 $p = 0.0001$	112.0 ± 5.1 $p = 0.07$	112.0 ± 5.1 $p = 0.0015$	121.4 ± 2 $p < 0.0001$	112.7 ± 2.6 $p = 0.001$	112.7 ± 2.6 $p = 0.01$	145.8 ± 9.0 $p = 0.001$	147.3 ± 5.4 $p < 0.0001$	147.3 ± 5.4 $p = 0.8$
VGAT	123.6 ± 2.9 $p < 0.0001$	105.3 ± 4 $p = 0.21$	105.3 ± 4 $p = 0.002$	125.3 ± 4.4 $p = 0.0003$	99.46 ± 9.2 $p = 0.9$	99.46 ± 9.2 $p = 0.01$	115.5 ± 3.5 $p = 0.0004$	106.2 ± 2.6 $p = 0.07$	106.2 ± 2.6 $p = 0.05$
GLUR1	89.70 ± 2.9 $p = 0.01$	105.8 ± 4.9 $p = 0.35$	105.8 ± 4.9 $p = 0.01$	93.71 ± 2.4 $p = 0.1$	93.44 ± 3.5 $p = 0.15$	93.44 ± 3.5 $p = 0.9$	67.25 ± 7.0 $p = 0.001$	66.55 ± 4.09 $p < 0.0001$	66.55 ± 4.09 $p = 0.9$
GLUR2	94.93 ± 2 $p = 0.12$	86.89 ± 2.6 $p = 0.001$	86.89 ± 2.6 $p = 0.02$	83.63 ± 1.9 $p = 0.0001$	95.20 ± 2.7 $p = 0.23$	95.20 ± 2.7 $p = 0.002$	63.61 ± 2.4 $p < 0.0001$	86.44 ± 3.1 $p = 0.01$	86.44 ± 3.1 $p < 0.0001$
NR1	92.60 ± 3 $p = 0.09$	92.16 ± 5.4 $p = 0.2$	92.16 ± 5.4 $p = 0.9$	88.38 ± 2.2 $p = 0.02$	107.8 ± 7.2 $p = 0.33$	107.8 ± 7.2 $p = 0.01$	96.90 ± 3.7 $p = 0.7$	94.35 ± 2.1 $p = 0.45$	94.35 ± 2.1 $p = 0.55$
NR2A	87.47 ± 2.1 $p = 0.001$	101.4 ± 9.3 $p = 0.9$	101.4 ± 9.3 $p = 0.1$	81.10 ± 1.9 $p = 0.001$	100.4 ± 7.0 $p = 0.9$	100.4 ± 7.0 $p = 0.01$	72.12 ± 4.7 $p = 0.002$	63.69 ± 3.4 $p = 0.0001$	63.69 ± 3.4 $p = 0.17$
VGLUT1	84.08 ± 1.8 $p = 0.0001$	107.8 ± 4.4 $p = 0.17$	107.8 ± 4.4 $p = 0.0001$	106.3 ± 1.9 $p = 0.04$	106.3 ± 2.8 $p = 0.1$	106.3 ± 2.8 $p = 0.9$	81.10 ± 4.5 $p = 0.01$	78.04 ± 4.6 $p = 0.005$	78.04 ± 4.6 $p = 0.6$
VGAT/VGLUT1	149.7 ± 4.5 $p < 0.0001$	98.33 ± 7.3 $p = 0.75$	98.33 ± 7.3 $p = 0.0001$	115.5 ± 4.8 $p = 0.02$	92.84 ± 7.4 $p = 0.36$	92.84 ± 7.4 $p = 0.02$	136.7 ± 9.8 $p = 0.008$	142.7 ± 9.3 $p = 0.002$	142.7 ± 9.3 $p = 0.6$
pCAMKII/CAMKII	86.32 ± 2.8 $p = 0.02$	72.58 ± 4.5 $p = 0.0005$	72.58 ± 4.5 $p = 0.02$	79.32 ± 3.4 $p = 0.0004$	99.12 ± 8.1 $p = 0.9$	99.12 ± 8.1 $p = 0.04$	89.30 ± 5.5 $p = 0.08$	93.70 ± 3.8 $p = 0.5$	93.70 ± 3.8 $p <= 0.1$

Protein levels relative to the levels detected in wild-type animals (WT: 100). Mean expression relative to WT level ± standard error of the mean (SEM), P-values for the t-test comparing transgenic (TG) vs. wild-type (WT) with treated transgenic (TG). (n = 10 for each genotype and for each treatment) CTX, cortex; HPC, Hippocampus; CRB, cerebellum. (Red indicates an increase, green a decrease, and blue no variation; changes were labeled for tendency toward significance with $p \leq 0.1$: pink for tendancy toward increase and pale green for tendancy toward decrease).*

analysis (6 weeks) for Ts65Dn; mice were euthanized at the end of treatment. Ts65Dn mice were euthanized at 6 months of age for molecular studies. POL60 contains green tea polyphenols with 27% EGCG, 42% other catechins (EC, ECG, EGC, and GC) with no effect on DYRK1A activity, and 8% caffeine; 1% sucrose was added. The placebo consisted of 1% sucrose in water. Both supplements were prepared fresh daily and offered *ad libitum*; water intake was measured on 5 days, and no difference was observed between the two groups. Decaffeinated MGTE (Life Extension) contains 45% EGCG and 53% other catechins. Solid food pellets containing MGTE were produced at a dose corresponding to 60 mg/kg/day EGCG; placebo was the ordinary solid diet. Caffeine-containing food pellets were produced at a dose corresponding to that absorbed from the POL60 supplement, i.e., 18 mg/kg/day. For each experiment, four groups of animals were used: wild-type (WT) and transgenic [TG; or trisomic (TS)] with placebo, WT and TG (or TS) with treatment.

Behavioral Analyses

To assess working memory impairment spontaneous alternation behavior was recorded in the Y-maze paradigm for the four groups of male WT and Tg/Ts animals as described in the Supplemental Methods section.

Tissue Collection

Male mice (3–4 months old for mBACtgDyrk1a were euthanized by decapitation, and brain tissue was rapidly removed. For immunoblotting, tissue was cooled on an ice block, dissected in less than 3 min, and snap-frozen in liquid nitrogen.

Immunoblotting

Immunoblotting was performed following standard Western or slot blot protocols. Antibodies were selected by Western blot for their suitability to slot blot analyses (Guedj et al., 2012; Souchet et al., 2014) (Supplementary Table 2). Digitized images of immunoblots were obtained using a LAS-3000 imaging system (Fuji Photo Film Co. Ltd.), and densitometry measurements were collected with an image analyzer (UnScan It software, Silk Scientific Inc.). Quantification of total proteins after Ponceau-S coloration was used as an internal control.

Statistical Analysis

For comparisons between groups analyzed by two, TG/WT, TG treated/WT, TG treated/TG, *t*-tests were performed. All graphs were plotted as mean ± SEM.

Data were considered significant when $p \leq 0.05$: in **Tables 1–6** a color code was used with red and green for significant increase and decrease respectively. A *p*-value of 0.06–0.10 was considered

TABLE 2 | Protein levels of markers of inhibition and excitation pathways for WT and Ts65Dn (TS) in cortex, hippocampus, and cerebellum following treatment with POL60 extract.

Inhibitors Comparison Markers	POL 60 -EGCG 67.5 mg/kg			POL 60 -EGCG 67.5 mg/kg			POL 60 -EGCG 67.5 mg/kg		
	TS/WT	TS*/WT	TS*/TS	TS/WT	TS*/WT	TS*/TS	TS/WT	TS*/WT	TS*/TS
	CTX			HPC			CRB		
DYRK1A	164.3 ± 20.3 $p = 0.01$	128.7 ± 10.9 $p = 0.05$	128.7 ± 10.9 $p = 0.12$	168.0 ± 24.2 $p = 0.02$	136.1 ± 10.9 $p = 0.03$	136.1 ± 10.9 $p = 0.2$	168.0 ± 24.2 $p = 0.02$	136.1 ± 10.9 $p = 0.03$	136.1 ± 10.9 $p = 0.21$
GAD67	144.4 ± 20 $p = 0.04$	84.34 ± 10.4 $p = 0.35$	84.34 ± 10.4 $p = 0.01$	189.3 ± 48.4 $p = 0.09$	109.0 ± 17.4 $p = 0.73$	109.0 ± 17.4 $p = 0.1$	152.2 ± 22.4 $p = 0.06$	147.9 ± 22 $p = 0.1$	147.9 ± 22 $p = 0.89$
GAD65	117.7 ± 7.5 $p = 0.06$	105.1 ± 5.2 $p = 0.45$	105.1 ± 5.2 $p = 0.21$	125.8 ± 14.7 $p = 0.09$	101.8 ± 4.8 $p = 0.57$	101.8 ± 4.8 $p = 0.1$	123.1 ± 10 $p = 0.09$	128.1 ± 10 $p = 0.05$	128.1 ± 10 $p = 0.7$
VGAT	213.3 ± 42.9 $p = 0.05$	87.47 ± 24.6 $p = 0.74$	87.47 ± 24.6 $p = 0.01$	129.1 ± 13.7 $p = 0.09$	104.7 ± 5.8 $p = 0.68$	104.7 ± 5.8 $p = 0.1$	144.3 ± 11.8 $p = 0.008$	121.6 ± 5 $p = 0.03$	121.6 ± 5 $p = 0.08$
GLUR1	84.17 ± 3.2 $p = 0.005$	104.6 ± 6.0 $p = 0.53$	104.6 ± 6.0 $p = 0.02$	105.8 ± 5.7 $p = 0.42$	98.69 ± 6.2 $p = 0.8$	98.69 ± 6.2 $p = 0.4$	86.66 ± 13.5 $p = 0.4$	79.61 ± 8.2 $p = 0.12$	79.61 ± 8.2 $p = 0.6$
GLUR2	77.05 ± 8.04 $p = 0.04$	97.08 ± 6.3 $p = 0.74$	97.08 ± 6.3 $p = 0.06$	106.4 ± 7.9 $p = 0.52$	107.5 ± 6.8 $p = 0.43$	107.5 ± 6.8 $p = 0.92$	98.93 ± 9.5 $p = 0.94$	98.18 ± 6.1 $p = 0.8$	98.18 ± 6.1 $p = 0.9$
NR1	105.2 ± 5.5 $p = 0.45$	100.1 ± 4 $p = 0.94$	100.1 ± 4 $p = 0.45$	85.25 ± 3.6 $p = 0.07$	96.9 ± 4.7 $p = 0.7$	96.9 ± 4.7 $p = 0.08$	85.25 ± 3.6 $p = 0.07$	96.95 ± 4.7 $p = 0.7$	96.95 ± 4.7 $p = 0.08$
NR2A	99.87 ± 3.1 $p = 0.98$	106.9 ± 3.6 $p = 0.27$	106.9 ± 3.6 $p = 0.17$	99.44 ± 8.1 $p = 0.7$	85.09 ± 7.4 $p = 0.26$	85.09 ± 7.4 $p = 0.21$	82.71 ± 3.1 $p = 0.01$	82.48 ± 4.4 $p = 0.02$	82.48 ± 4.4 $p = 0.9$
VGLUT1	88.45 ± 2.18 $p = 0.01$	103.7 ± 3.7 $p = 0.5$	103.7 ± 3.7 $p = 0.008$	91.24 ± 2.4 $p = 0.02$	100.5 ± 3.7 $p = 0.9$	100.5 ± 3.7 $p = 0.08$	106.1 ± 7.4 $p = 0.36$	102.3 ± 5 $p = 0.47$	102.3 ± 5 $p = 0.6$
VGAT/VGLUT1	247.4 ± 55.5 $p = 0.04$	87.86 ± 25.9 $p = 0.73$	87.86 ± 25.9 $p = 0.01$	142.5 ± 16.5 $p = 0.03$	106.7 ± 4.3 $p = 0.44$	106.7 ± 4.3 $p = 0.04$	138.5 ± 12.1 $p = 0.01$	118.9 ± 7.1 $p = 0.05$	118.9 ± 7.1 $p = 0.18$
pCAMKII/CAMKII	50.32 ± 12.6 $p = 0.05$	102.1 ± 17.9 $p = 0.9$	102.1 ± 17.9 $p = 0.05$	59.08 ± 14.1 $p = 0.08$	124.1 ± 20.1 $p = 0.36$	124.1 ± 20.1 $p = 0.03$	75.59 ± 5.3 $p = 0.05$	66.06 ± 3.5 $p = 0.03$	66.06 ± 3.5 $p = 0.15$

Protein levels relative to the levels detected in wild-type animals (WT: 100). Mean expression relative to WT level ± standard error of the mean (SEM), P-values for the t-test comparing trisomic (TS) vs. wild-type (WT) with treated trisomic (TS). (n = 10) CTX, cortex; HPC, Hippocampus; CRB, cerebellum. (Red indicates an increase, green a decrease, and blue no variation; changes were labeled for tendency toward significance with $p \leq 0.1$: pink for tendancy toward increase and pale green for tendancy toward decrease).*

to indicate a strong statistical tendency due to the small sample size: in **Tables 1–6** tendancy to an increase was coded in pink and tendancy to a decrease was coded in pale green.

Behavioral analyses were performed using the Mann-Whitney test as the non-normality of data precluded the use of parametric statistics (e.g., analysis of variance). All statistical analyses were performed using GraphPad6 software package.

Results

Effects of POL60 Extract Treatment

mBACtgDyrk1a Mice

To better understand the previously observed effects of EGCG treatment on improving behavioral outcomes in DS mouse models, as well as humans with DS, adult WT and TG mice were treated for 1 month with POL60 diluted in water, with an average consumption of 3–5 mL per day, corresponding to a dose of 60 mg/kg EGCG. Markers involved in both GABAergic and glutaminergic synaptic plasticity pathways and previously characterized in various DYRK1A murine models were assessed by immunoblot to characterize the impact of treatment on E/I balance (**Figure 1**, **Table 1**). In the cortex, hippocampus, and cerebellum, overexpression of DYRK1A generally promoted higher protein levels of GABAergic markers in Tg mice compared to WT, but these levels decreased following EGCG treatment. GAD67 expression was altered in all three brain regions, while VGAT was affected only in cortex and hippocampus. In contrast, protein levels of glutaminergic markers GLUR1, NR1, NR2a, and VGLUT1 were lower in cortex of Tg mice, but their levels returned to that of WT following treatment. Similar changes were observed in the hippocampus, with the exception of VGLUT1, which was not altered in the hippocampus of transgenic animals. We observed a weaker correction of GABAergic markers in the cerebellum than in other brain regions, and no correction of glutaminergic markers in the cerebellum. The ratio of PCAMKII/CAMKII, an indicator of LTP status, was lower in TG mice in all three brain regions analyzed; a rescue effect following EGCG treatment was observed only in the hippocampus.

Ts65Dn Mice

The same POL60 oral treatment was applied to adult Ts65Dn animals to assess the effect of treatment in the trisomic context. After 1 month of treatment, short-term memory was assessed using spontaneous alternation in the Y maze (**Figure 2**). Percentage of alternation was lower in Ts65Dn animals than in WT ($P = 0.0002$). However, this difference was rescued by

TABLE 3 | Protein levels of markers of inhibition and excitation pathways for WT and mBACtgDyrk1a (Tg) in cortex following treatment with 3 doses of MGTE extract.

Inhibitors Comparison Markers	TG/WT	MGTE- EGCG I- 10 mg/kg		MGTE- EGCG II- 60 mg/kg		MGTE- EGCGIII- 360 mg/kg	
		TG*/WT	TG*/TG	TG*/WT	TG*/TG	TG*/WT	TG*/TG
Cortex							
DYRK1A	162.2 ± 3.3 $p < 0.0001$	139.1 ± 7.9 $p < 0.0001$	139.1 ± 7.9 $p = 0.01$	148.2 ± 8.0 $p < 0.0001$	148.2 ± 8.0 $p = 0.1$	193.4 ± 14.0 $p < 0.0001$	193.4 ± 14.0 $p = 0.02$
GAD67	131.9 ± 6.6 $p = 0.0006$	133.7 ± 10.8 $p = 0.0007$	133.7 ± 10.8 $p = 0.8$	131.2 ± 5.7 $p = 0.0003$	131.2 ± 5.7 $p = 0.9$	159.5 ± 15.6 $p < 0.0001$	159.5 ± 15.6 $p = 0.08$
GAD65	136.4 ± 4 $p < 0.0001$	114.3 ± 7.0 $p = 0.04$	114.3 ± 7.0 $p = 0.01$	115.2 ± 6.4 $p = 0.05$	115.2 ± 6.4 $p = 0.01$	131.3 ± 8.9 $p = 0.002$	131.3 ± 8.9 $p = 0.56$
VGAT	123.6 ± 2.9 $p < 0.0001$	99.98 ± 5.3 $p = 0.9$	99.98 ± 5.3 $p = 0.0009$	94.53 ± 3.7 $p = 0.35$	94.53 ± 3.7 $p < 0.0001$	102.6 ± 3.7 $p = 0.7$	102.6 ± 3.7 $p = 0.0004$
NR1	92.60 ± 3 $p = 0.09$	99.53 ± 5.3 $p = 0.87$	99.53 ± 5.3 $p = 0.1$	91.98 ± 4.9 $p = 0.1$	91.98 ± 4.9 $p = 0.9$	85.50 ± 3.3 $p = 0.006$	85.50 ± 3.3 $p = 0.1$
NR2A	87.47 ± 2.1 $p = 0.001$	101.8 ± 4.5 $p = 0.8$	101.8 ± 4.5 $p = 0.007$	98.00 ± 2.6 $p = 0.6$	98.00 ± 2.6 $p = 0.006$	90.54 ± 2.6 $p = 0.01$	90.54 ± 2.6 $p = 0.37$
GLUR1	89.70 ± 2.9 $p = 0.01$	94.90 ± 4.9 $p = 0.39$	94.90 ± 4.9 $p = 0.36$	106.7 ± 4.5 $p = 0.25$	106.7 ± 4.5 $p = 0.006$	111.3 ± 6 $p = 0.15$	111.3 ± 6 $p = 0.005$
GLUR2	94.93 ± 2 $p = 0.1$	91.06 ± 2.4 $p = 0.12$	91.06 ± 2.4 $p = 0.24$	98.26 ± 4.2 $p = 0.8$	98.26 ± 4.2 $p = 0.5$	88.80 ± 1.9 $p = 0.008$	88.80 ± 1.9 $p = 0.05$
VGLUT1	84.08 ± 1.8 $p = 0.0001$	96.27 ± 3.6 $p = 0.34$	96.27 ± 3.6 $p = 0.005$	95.16 ± 3 $p = 0.19$	95.16 ± 3 $p = 0.005$	101.2 ± 5.9 $p = 0.8$	101.2 ± 5.9 $p = 0.006$
VGAT/VGLUT1	149.7 ± 4.5 $p < 0.0001$	118.7 ± 2.1 $p = 0.02$	118.7 ± 2.1 $p = 0.02$	110.0 ± 4.9 $p = 0.22$	110.0 ± 4.9 $p = 0.0001$	115.8 ± 7.3 $p = 0.08$	115.8 ± 7.3 $p = 0.01$
*p*CAMKII/CAMKII	86.32 ± 2.8 $p = 0.01$	80.14 ± 11.7 $p = 0.08$	80.14 ± 11.7 $p = 0.48$	52.77 ± 5.3 $p < 0.0001$	52.77 ± 5.3 $p < 0.0001$	102.1 ± 11.8 $p = 0.79$	102.1 ± 11.8 $p = 0.08$

Protein levels relative to the levels detected in wild-type animals (WT: 100). Mean expression relative to WT level ± standard error of the mean (SEM), P-values for the t-test comparing transgenic (TG) vs. wild-type (WT) with treated transgenic (TG). (n = 10). (Red indicates an increase, green a decrease, and blue no variation; changes were labeled for tendency toward significance with $p \leq 0.1$: pink for tendancy toward increase and pale green for tendancy toward decrease).*

POL60, with a significant increase of spontaneous alternation after treatment ($p = 0.0022$).

After these behavioral assessments, mice were euthanized and brains were collected and analyzed as for mBACtgDyrk1a mice. As was observed in the TG mice, the levels of GABAergic markers were higher in all three brain regions, and levels of glutaminergic markers were lower (with the exception of GLUR1 and GLUR2 in hippocampus) in TS mice compared to WT (**Table 2**). However, treatment with POL60 resulted in rescued levels of GABAergic and glutaminergic markers in cortex and hippocampus. Further, the ratio of pCAMKII/CAMKII, which was significantly lower in Ts65Dn mice, was rescued by treatment. In contrast, treatment did not modify the alterations observed in the cerebellum (**Table 2**).

Treatment with MGTE in mBACtgDyrk1a Mice: Dose Effects and Behavioral Rescue

For translational purposes, we chose to continue our analyses with an extract used for food supplementation or direct consumption in humans: MGTE, which contains 45% EGCG and three other catechins. To select the right dose of EGCG, the effects of three doses were compared: a dose 6 times lower than the EGCG doses previously used (dose I = 10 mg/kg); a dose similar to the previous experiments (dose II = 60 mg/kg); and a dose 6 times higher than the intermediate dose (dose III = 360 mg/kg). WT and Tg adult (3–4 months) mice were treated with MGTE-supplemented solid food with an average consumption of 3–5 g per day. The same GABAergic and glutaminergic markers in cortex, hippocampus, and cerebellum were assessed following euthanization. To determine the true defect due to transgenesis, we used the average values of expression levels obtained for 10 WT/10 TG (3–5 experiments for each marker), and these values were compared to the values obtained for treated animals ($n = 10$ for WT and TG). In cortex (**Table 3**) we observed a tendency toward lower DYRK1A and GAD65 following treatment with low and intermediate doses. GAD67 levels were not modified by the low and intermediate doses, but were significantly higher after high-dose treatment. DYRK1A was significantly lower following high-dose treatment. VGAT and VGLUT1 levels were rescued by all three doses. NR1 and NR2A levels were rescued by low-dose treatment, and NR2A also by the intermediate dose. PCAMKII/CAMKII was rescued only by dose III. Thus, in cortex the intermediate dose (dose II) appeared to be the best compromise to rescue normal levels of VGAT/VGLUT1 and to avoid the increase in GAD67 and DYRK1A levels observed with dose III.

TABLE 4 | Protein levels of markers of inhibition and excitation pathways for WT and mBACtgDyrk1a (TG) in hippocampus following treatment with 3 doses of MGTE extract.

Inhibitors Comparison Markers	TG/WT	MGTE-EGCG I- 10 mg/kg		MGTE-EGCG II- 60 mg/kg		MGTE-EGCGIII- 360 mg/kg	
		TG*/WT	TG*/TG	TG*/WT	TG*/TG	TG*/WT	TG*/TG
Hippocampus							
DYRK1A	199.8 ± 9.2 $p < 0.0001$	164.9 ± 4.2 $p < 0.0001$	164.9 ± 4.2 $p = 0.004$	129.5 ± 5.2 $p = 0.002$	129.5 ± 5.2 $p = 0.0001$	164.4 ± 3.7 $p < 0.0001$	164.4 ± 3.7 $p = 0.008$
GAD67	142.2 ± 5.4 $p < 0.0001$	123.5 ± 8.3 $p = 0.01$	123.5 ± 8.3 $p = 0.07$	123.8 ± 5.1 $p = 0.001$	123.8 ± 5.1 $p = 0.02$	148.4 ± 8.6 $p < 0.0001$	148.4 ± 8.6 $p = 0.46$
GAD65	121.4 ± 2 $p < 0.0001$	122.1 ± 5.4 $p < 0.0001$	122.1 ± 5.4 $p = 0.8$	118.2 ± 4.8 $p = 0.002$	118.2 ± 4.8 $p = 0.7$	117.1 ± 4.4 $p = 0.0006$	117.1 ± 4.4 $p = 0.7$
VGAT	125.3 ± 4.4 $p = 0.0003$	95.55 ± 7.2 $p = 0.47$	95.55 ± 7.2 $p = 0.002$	102.9 ± 5.3 $p = 0.57$	102.9 ± 5.3 $p = 0.005$	101.2 ± 5.4 $p = 0.8$	101.2 ± 5.4 $p = 0.003$
GLUR1	93.71 ± 2.4 $p = 0.1$	83.72 ± 2.4 $p = 0.003$	83.72 ± 2.4 $p = 0.01$	94.06 ± 6.0 $p = 0.31$	94.06 ± 6.0 $p = 0.9$	92.94 ± 2.5 $p = 0.24$	92.94 ± 2.5 $p = 0.43$
GLUR2	83.63 ± 1.9 $p = 0.0001$	90.28 ± 2.6 $p = 0.1$	90.28 ± 2.6 $p = 0.05$	99.42 ± 1.9 $p = 0.9$	99.42 ± 1.9 $p = 0.0001$	90.49 ± 4.8 $p = 0.15$	90.49 ± 4.8 $p = 0.26$
NR1	88.38 ± 2.2 $p = 0.02$	108.3 ± 6.9 $p = 0.31$	108.3 ± 6.9 $p = 0.01$	113.2 ± 7.1 $p = 0.1$	113.2 ± 7.1 $p = 0.005$	133.9 ± 7.2 $p = 0.0005$	133.9 ± 7.2 $p < 0.0001$
NR2A	81.10 ± 1.9 $p = 0.001$	82.25 ± 4.4 $p = 0.01$	82.25 ± 4.4 $p = 0.78$	121.8 ± 6.7 $p = 0.02$	121.8 ± 6.7 $p < 0.0001$	110.8 ± 4.6 $p = 0.29$	110.8 ± 4.6 $p < 0.0001$
VGLUT1	106.4 ± 3.1 $p = 0.05$	98.46 ± 5.7 $p = 0.61$	98.46 ± 5.7 $p = 0.23$	93.32 ± 4.3 $p = 0.05$	93.32 ± 4.3 $p = 0.02$	98.60 ± 5.0 $p = 0.7$	98.60 ± 5.0 $p = 0.24$
VGAT/VGLUT1	115.5 ± 4.8 $p = 0.02$	89.13 ± 4.8 $p = 0.07$	89.13 ± 4.8 $p = 0.001$	111.2 ± 3.9 $p = 0.05$	111.2 ± 3.9 $p = 0.5$	103.8 ± 5.8 $p = 0.58$	103.8 ± 5.8 $p = 0.1$
*p*CAMKII/CAMKII	79.33 ± 3.3 $p = 0.0004$	75.63 ± 6.6 $p = 0.005$	75.63 ± 6.6 $p = 0.59$	99.77 ± 12.3 $p = 0.89$	99.77 ± 12.3 $p = 0.1$	96.93 ± 7.4 $p = 0.81$	96.93 ± 7.4 $p = 0.04$

Protein levels relative to the levels detected in wild-type animals (WT: 100). Mean expression relative to WT level ± standard error of the mean (SEM), P-values for the t-test comparing transgenic (TG) vs. wild-type (WT) with treated transgenic (TG). (n = 10). (Red indicates an increase, green a decrease, and blue no variation; changes were labeled for tendency toward significance with $p \leq 0.1$: pink for tendancy toward increase and pale green for tendancy toward decrease).*

In hippocampus (**Table 4**), where the basal level of overexpression of DYRK1A was high, we observed lower, but not normal, DYRK1A levels after treatment of TG mice with the three doses. Neither GAD67 nor GAD65 were modified by the treatment. However, VGAT was rescued to a normal level at all three EGCG doses. Notably, the levels of three markers of the glutaminergic pathway, GLUR2, NR1, and NR2A, were corrected when using treatment II. For NR2A, this correction significantly exceeded the normal level.

In cerebellum (**Table 5**), the treatments induced significant decreases in levels of DYRK1A at doses I and II, and increases in GAD67 at doses II and III. VGAT1 was not modified by the treatment. The only rescuing effect was observed for GLUR1 and GLUR2, at doses II and III. The ratio of VGAT/VGLUT1, which is higher in untreated transgenic mice, was significantly increased by the three MGTE doses.

We used a spontaneous alternation paradigm (similar to the Y–maze experiment performed with POL60 treated Ts65Dn mice) to assess the effects of DYRK1A overexpression on short-term spatial working memory. We found that the exploratory activity in the Y-maze was affected by Dyrk1a overexpression: the total number of arm entries was higher in mBACtgDyrk1a mice compared with wild type animals in both conditions of treatment (placebo and MGTE, $p = 0.012$ and $p = 0.009$ respectively).

The rate of spontaneous alternation (visiting each arm in turn) was affected by genotype: mBACtgDyrk1a mice alternated less than wild-type mice ($p = 0.017$, **Figure 3**). Noteworthy, treatment improved the rate of spontaneous alternation of mBACtgDryk1a mice ($p = 0.03$).

Treatment with Caffeine in mBACtgDyrk1a Mice: Molecular Effects

To explain the differences observed in the corrections of GAD67 levels between POL60 treatment and MGTE treatment, we hypothesized an effect of the caffeine contained in green tea and present in POL60 extract. We designed a caffeine-supplemented solid diet alone with a caffeine dose (18 mg/kg) equivalent to the dose given to mice treated with POL60. After 1 month of treatment, adult mBACtgDyrk1a and wild-type mice animals were euthanized and brains collected and analyzed (**Table 6**). In cortex, we observed significantly decreased levels of markers of GABAergic neurotransmission: levels of GAD67 and VGAT1 and the ratio of VGAT/VGLUT1 were partially rescued. In hippocampus, rescue of these markers was complete. In contrast, in cortex and hippocampus, markers of glutaminergic neurotransmission, GLUR1 and GLUR2, remained at low levels after treatment, and the levels of NR1 and NR2A were further decreased after treatment.

TABLE 5 | Protein levels of markers of inhibition and excitation pathways for WT and mBACtgDyrk1a (Tg) in cerebellum following treatment with 3 doses of MGTE extract.

Treatment		MGTE-EGCG I- 10 mg/kg		MGTE-EGCG II- 60 mg/kg		MGTE-EGCGIII- 360 mg/kg	
Markers	TG/WT	TG*/WT	TG*/TG	TG*/WT	TG*/TG	TG*/WT	TG*/TG
Cerebellum							
DYRK1A	171.0 ± 9 $p < 0.0001$	136.5 ± 9.7 $p = 0.003$	136.5 ± 9.7 $p = 0.01$	139.4 ± 7.0 $p = 0.0002$	139.4 ± 7.0 $p = 0.01$	155.6 ± 13.9 $p < 0.0001$	155.6 ± 13.9 $p = 0.35$
GAD67	159.8 ± 6.7 $p = 0.0001$	150.6 ± 13.0 $p = 0.002$	150.6 ± 13.0 $p = 0.5$	181.4 ± 10.9 $p = 0.0001$	181.4 ± 10.9 $p = 0.1$	188.6 ± 17.3 $p < 0.0001$	188.6 ± 17.3 $p = 0.1$
GAD65	139.6 ± 5.1 $p = 0.0001$	138.1 ± 5.6 $p < 0.0001$	138.1 ± 5.6 $p = 0.85$	148.2 ± 9.05 $p = 0.0002$	148.2 ± 9.05 $p = 0.41$	168.2 ± 13.6 $p = 0.0001$	168.2 ± 13.6 $p = 0.04$
VGAT	122.6 ± 3.2 $p < 0.0001$	136.6 ± 4.0 $p < 0.0001$	136.6 ± 4.0 $p = 0.68$	133.6 ± 3.6 $p < 0.0001$	133.6 ± 3.6 $p = 0.04$	135.3 ± 3.9 $p < 0.0001$	135.3 ± 3.9 $p = 0.02$
GLUR1	82.87 ± 2.6 $p = 0.0005$	98.01 ± 5.3 $p = 0.74$	98.01 ± 5.3 $p = 0.01$	92.62 ± 5.3 $p = 0.21$	92.62 ± 5.3 $p = 0.1$	100.7 ± 6.2 $p = 0.91$	100.7 ± 6.2 $p = 0.01$
GLUR2	87.94 ± 2.7 $p = 0.009$	103.6 ± 3.6 $p = 0.48$	103.6 ± 3.6 $p = 0.003$	107.2 ± 2.8 $p = 0.09$	107.2 ± 2.8 $p = 0.0001$	111.7 ± 4.7 $p = 0.04$	111.7 ± 4.7 $p = 0.0005$
NR1	98.84 ± 1.6 $p = 0.7$	90.71 ± 2.6 $p = 0.08$	90.71 ± 2.6 $p = 0.01$	94.11 ± 4.1 $p = 0.3$	94.11 ± 4.1 $p = 0.28$	94.18 ± 3.5	94.18 ± 3.5 $p = 0.2$
NR2A	85.2 ± 2.9 $p = 0.001$	94.08 ± 5.6 $p = 0.4$	94.08 ± 5.6 $p = 0.15$	89.99 ± 3.6 $p = 0.04$	89.99 ± 3.6 $p = 0.36$	91.1 ± 3.8 $p = 0.07$	91.15 ± 3.8 $p = 0.31$
VGLUT1	84.5 ± 2 $p < 0.0001$	83.82 ± 2.7 $p = 0.0005$	83.82 ± 2.7 $p = 0.66$	81.72 ± 3.0 $p = 0.0001$	81.72 ± 3.0 $p < 0.33$	77.67 ± 2.1 $p < 0.0001$	77.67 ± 2.1 $p = 0.07$
VGAT/VGLUT1	141.6 ± 5.1 $p < 0.0001$	161.1 ± 3.7 $p < 0.0001$	161.1 ± 3.7 $p = 0.008$	162.0 ± 8.4 $p < 0.0001$	162.0 ± 8.4 $p = 0.06$	173.1 ± 7.8 $p < 0.0001$	173.1 ± 7.8 $p = 0.004$

Protein levels relative to the levels detected in wild-type animals (WT: 100). Mean expression relative to WT level ± standard error of the mean (SEM), P-values for the t-test comparing transgenic (TG) vs. wild-type (WT) with treated transgenic (TG). (n = 10). (Red indicates an increase, green a decrease, and blue no variation; changes were labeled for tendency toward significance with $p \leq 0.1$: pink for tendancy toward increase and pale green for tendancy toward decrease).*

Discussion

We found that DYRK1A protein level is associated with expression levels of proteins involved in synaptic plasticity. Specifically, enzymes involved in decarboxylation of glutamate to produce GABA and in vesicular transport of GABA are found at higher levels in mice with three copies of *Dyrk1a*. In *Dyrk1a* single-copy mice, only GABA-producing enzymes are detected at lower levels than in WT (Souchet et al., 2014); the increase in VGAT1 in hippocampus and cortex may be compensating for these reductions. In contrast, in the cerebellum GAD67 and VGAT levels were changed in the same direction. Thus, molecular data suggest that increasing *Dyrk1a* dosage induces activation of the GABA pathway with an increased production and transport of GABA; decreasing the level of *Dyrk1a* induces a decrease in both GADs. These molecular changes offer mechanistic support for behavioral phenotypes observed in mouse models. In Ts65Dn, excessive GABAergic neurotransmission results in local over-inhibition of hippocampal circuits, which dampens hippocampal synaptic plasticity and contributes to cognitive impairments; treatment with several GABA-A receptor antagonists results in increased plasticity and improved memory deficits in Ts65Dn mice (Fernandez et al., 2007). Deficits in cognition and synaptic plasticity in Ts65Dn are also ameliorated by a selective inverse agonist of GABA-A receptor α5 subtype (Braudeau et al., 2011) or by GABA-B receptor antagonists (Kleschevnikov et al., 2012). Reducing GABA-A α5 receptor-mediated inhibition normalizes the high density of GABAergic synapse markers in the molecular layer of the hippocampus of TS mice (Martinez-Cue et al., 2013).

Here we report the phenotypic rescues observed in adult murine models of DS after a 1-month oral treatment with green tea extracts containing EGCG. We propose a molecular clue to understand the mechanisms of increased inhibition in DS and for the correcting effects of EGCG.

EGCG-containing Extracts Rescue Components of E/I Balance

For the first time, we compared the effect of an inhibitor of DYRK1A, EGCG, on the molecular phenotypes of adult mBACtgDyrk1a mice and Ts65Dn mice. We previously showed the gene dosage effect of *Dyrk1a* on GABAergic and glutaminergic pathways in models with increased DYRK1A expression and decreased DYRK1A expression. DYRK1A dose has an impact on the levels of GABA-synthesizing enzymes GAD67 and GAD65, but also on GABA transporter VGAT. DYRK1A dose also affects excitatory processes and modifies levels of glutamate receptors GLUR1 and GLUR2, of a glutamate transporter VGLUT1, and components of the NMDA receptor,

TABLE 6 | Protein levels of markers of inhibition and excitation pathways for WT and mBACtgDyrk1a (Tg) in cortex and hippocampus following treatment with caffeine.

Inhibitors Comparison Markers		Caffeine			Caffeine	
	TG/WT	TG*/WT	TG*/TG	TG/WT	TG*/WT	TG*/TG
	CTX			HPC		
DYRK1A	162.2 ± 3.3 $p < 0.0003$	180.1 ± 8.1 $p < 0.0001$	180.1 ± 8.1 $p = 0.8$	199.8 ± 9.2 $p < 0.0001$	149.4 ± 14 $p < 0.0001$	149.4 ± 14 $p = 0.006$
GAD67	131.9 ± 6.6 $p = 0.0006$	110.3 ± 7.8 $p = 0.22$	110.3 ± 7 $p = 0.05$	142.2 ± 5.4 $p < 0.0001$	103.7 ± 9.2 $p = 0.8$	103.7 ± 9.2 $p = 0.001$
VGAT	123.6 ± 2.9 $p < 0.0001$	109.3 ± 7.9 $p = 0.2$	109.3 ± 7.9 $p = 0.07$	125.3 ± 4.4 $p = 0.0003$	99.46 ± 9.2 $p = 0.9$	99.46 ± 9.2 $p = 0.008$
GLUR1	89.70 ± 2.9 $p = 0.01$	85.94 ± 2.8 $p = 0.002$	85.94 ± 2.8 $p = 0.39$	93.71 ± 2.4 $p = 0.1$	93.44 ± 3.5 $p = 0.1$	93.44 ± 3.5 $p = 0.9$
GLUR2	94.93 ± 2 $p = 0.1$	96.86 ± 5 $p = 0.58$	96.86 ± 5 $p = 0.9$	83.63 ± 1.9 $p = 0.0001$	85.94 ± 2.8 $p = 0.003$	85.94 ± 2.8 $p = 0.5$
NR1	92.60 ± 3 $p = 0.09$	85.18 ± 3.8 $p = 0.01$	85.18 ± 3.8 $p = 0.1$	88.38 ± 2.2 $p = 0.02$	68.20 ± 5.6 $p = 0.0004$	68.20 ± 5.6 $p = 0.001$
NR2A	87.47 ± 2.1 $p = 0.001$	69.23 ± 4 $p < 0.0001$	69.23 ± 4 $p = 0.0005$	81.10 ± 1.9 $p = 0.001$	76.56 ± 7.3 $p = 0.01$	76.56 ± 7.3 $p = 0.49$
VGLUT1	84.08 ± 1.8 $p < 0.0001$	91.54 ± 2.7 $p = 0.01$	91.54 ± 2.7 $p = 0.03$	106.3 ± 1.9 $p = 0.04$	98.12 ± 4.4 $p = 0.5$	98.12 ± 4.4 $p = 0.15$
VGAT/VGLUT1	149.7 ± 4.5 $p < 0.0001$	125.8 ± 9.2 $p = 0.01$	125.8 ± 9.2 $p = 0.02$	115.5 ± 4.8 $p = 0.02$	96.09 ± 7 $p = 0.56$	96.09 ± 7 $p = 0.03$
pCAMKII/CAMKII	86.35 ± 3.3 $p = 0.02$	100.7 ± 8.6 $p = 0.9$	100.7 ± 8.6 $p = 0.1$	79.32 ± 3.4 $p = 0.0004$	115.9 ± 5.5 $p = 0.01$	115.9 ± 5.5 $p < 0.0001$

Protein levels relative to the levels detected in wild-type animals (WT: 100). Mean expression relative to WT level ± standard error of the mean (SEM), P-values for the t-test comparing transgenic (TG) vs. wild-type (WT) with treated transgenic (TG). (n = 10) CTX, cortex; HPC, Hippocampus; CRB, cerebellum. (Red indicates an increase, green a decrease, and blue no variation; changes were labeled for tendency toward significance with $p \leq 0.1$: pink for tendancy toward increase and pale green for tendancy toward decrease).*

NR1 and NR2A. Further, overexpression of DYRK1A reduces the activation of CAMKII, which is accompanied by anomalous NMDAR-mediated long-term potentiation (Thomazeau et al., 2014). Treatment with POL60 (27% EGCG) and MGTE (45% EGCG), given at EGCG equivalent doses (60 mg/kg) produced corrections in the levels of most of these markers, conducive to a rescue of E/I balance in agreement with the rescue of working memory. Even if we observed some differences in molecular alterations between mBACtgDyrk1a and Ts65Dn mice, which might be linked to the additional gene context in Ts65Dn, alterations were in the same direction and were corrected by POL60 treatment in similar ways in both models.

Molecular Effects are Brain-region Dependent

Most of the E/I markers varied in the same direction between WT and transgenic or WT and trisomic mice when different brain regions were compared. However, in mBACtgDyrk1a VGLUT1 was decreased in cortex and cerebellum but slightly increased in hippocampus; in Ts65Dn, the same marker was decreased in cortex and hippocampus and showed a non-significant increase in cerebellum. POL60 treatment corrected these alterations in both models, with the exception of GLUR2 and pCAMKII/CAMKII in cortex and in hippocampus of mBACtgDyrk1a. In cerebellum, in mBACtgDyrk1a we observed only partial correction for GAD67 and VGAT, and in Ts65Dn we observed only a partial correction for VGAT. This difference for cerebellum is intriguing and might be due to a reduced accessibility of the drug although this hypothesis is not compatible with the effect of MGTE on DYRK1A or GLUR1-GLUR2 levels; therefore these differences are most probably due to the presence of different regulatory mechanisms in cerebellum.

EGCG Molecular Effects are Dose-dependent

To further analyze molecular effects of EGCG treatment we compared three doses of decaffeinated MGTE compound already used in previous mouse studies and in a pilot trial; the intermediate EGCG dose was similar to the dose used for the POL60 study. In cortex the effect of the lower and intermediate doses on DYRK1A and markers from the GABA system were similar, with a partial decrease for DYRK1A and GAD65 and a complete correction for VGAT. At the highest dose, opposite effects were observed for DYRK1A, accompanied by increased GAD67, potentially exacerbating E/I imbalance. In hippocampus, low and intermediate doses induced a partial rescue of DYRK1A and GAD67. Interestingly, the stability of DYRK1A has been associated with autophosphorylation (Himpel et al., 2001), an activity that might be decreased in the presence of inhibitors. In cerebellum, the intermediate and high doses

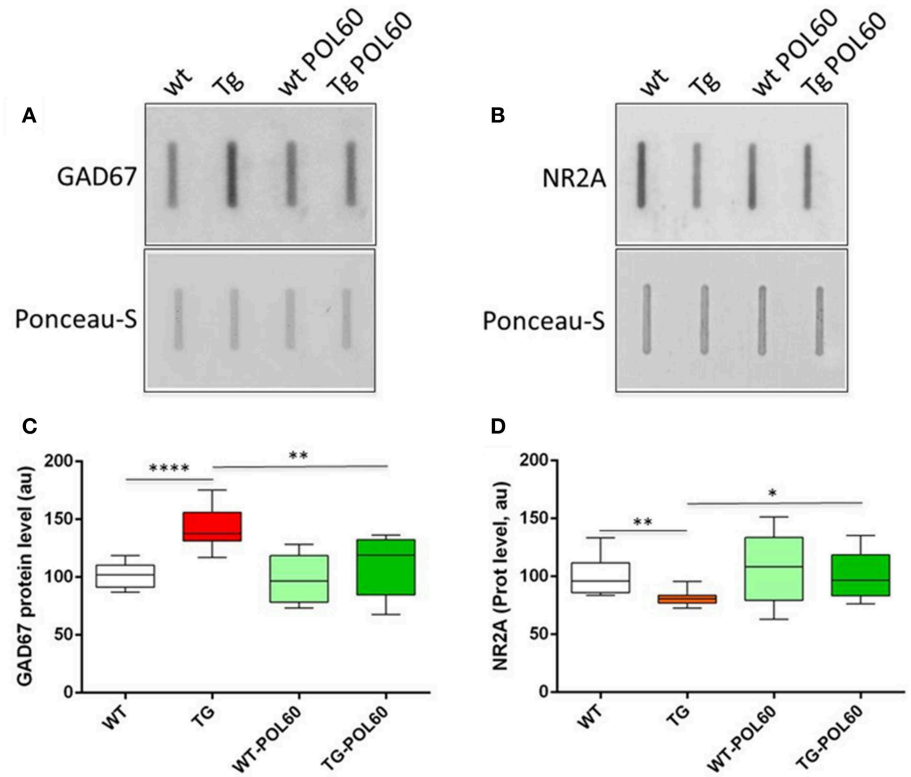

FIGURE 1 | Effect of long-term POL60 treatment on GAD67 and NR2A levels in hippocampus: Immunoblotting of proteins from wt and mBACtgDyrk1a hippocampus treated with placebo or POL60 for **(A)** GAD67 and **(B)** NR2A. Ponceau-S coloration was used to assess total protein levels. Below: boxplots of expression relative to WT placebo (WT) for **(C)** GAD67 and **(D)** NR2A. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$.

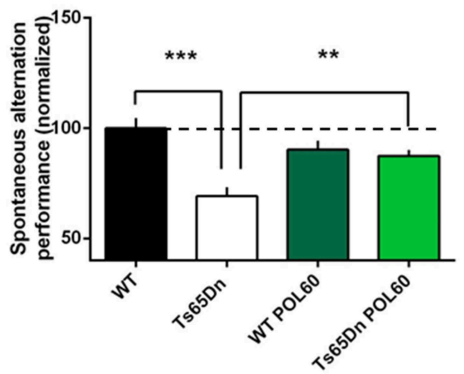

FIGURE 2 | Effect of long-term POL60 treatment on working memory in Ts65Dn mice: Per cent of alternation was assessed in four groups of mice: WT and Ts administered placebo, $n = 12$; and WT and Ts with POL60 treatment, $n = 8$. The data have been normalized to the baseline level of performance of wild-type mice fed with water (dotted line). Ts65Dn mice displayed a very significant decrease of alternation under placebo condition. POL60 treatment produced improved alternation performance in the Ts65Dn mice. $^{***}p < 0.001$, $^{**}p < 0.01$.

induced increased GAD67 and ratio of VGAT1/VGLUT1. For the glutaminergic pathway in cortex, corrections NR1, NR2A, and VGLUT1 were already present for the lower dose. These findings suggest that a dose below or close to the intermediate dose is the best choice for further studies.

EGCG Molecular Effects are Extract-dependent and Differences are Explained by the Presence of Caffeine in POL60 Extracts

We observed, particularly in cortex, that a low or intermediate dose of MGTE does not change the level of GAD67, despite the rescue of GAD67 levels following treatment with POL60 at an equivalent dose of EGCG. Comparison of composition of POL60 and decaffeinated MGTE extracts indicates that POL60 contains a dose corresponding to an 18 mg/kg caffeine diet. Therefore, hypothesizing that caffeine partially mediates the effects of POL60, we fed mBACtgDyrk1a adult mice an 18 mg/kg caffeine diet in solid food. Brain synaptic marker analysis revealed that this dose of caffeine was sufficient to induce a partial rescue of GAD67 and VGAT levels in cortex, and a complete rescue of these markers in hippocampus. Glutaminergic markers were not rescued by this treatment. The mode of action of caffeine is unknown, but might involve an effect on GAD67 via A2A receptors (Carta et al., 2002). Caffeine has no effect on glutaminergic markers; however, it rescues alterations of pCAMKII/CAMKII ratio in cortex and induces an increase in hippocampus in comparison with WT.

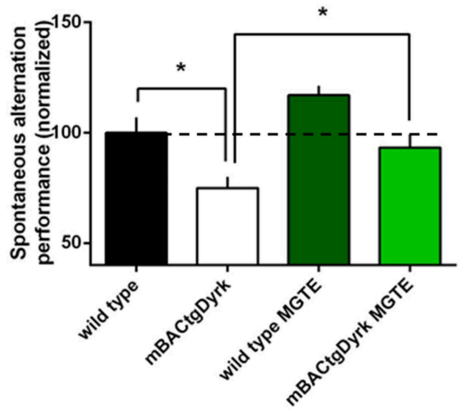

FIGURE 3 | Cognitive performance of GTE (or MGTE)-treated mBACtgDyrk1a in the Y maze. The effects of *Dyrk1A* overexpression on short-term spatial memory were assessed in a spontaneous alternation paradigm in a Y–maze. The data have been normalized to the baseline level of performance of wild-type mice fed with water (dotted line). One mouse from the BACtgDyrk1a group was excluded from statistical analysis because of abnormally high levels of locomotor activity and associated erratic exploration, precluding assessment of memory scores. The rate of spontaneous alternation (visiting each arm in turn) was affected by genotype: mBACtgDyrk1a mice alternated less than wild-type mice ($P < 0.05$). Treatment with MGTE significantly improved performance of mBACtgDyrk1a mice. $^{*}p < 0.05$.

EGCG-containing Extracts Rescue Short-term Memory in Transgenic and Trisomic Models

We previously reported that EGCG treatment can rescue spatial learning (De la Torre et al., 2014) and object recognition memory (Guedj et al., 2009; De la Torre et al., 2014) deficits in Ts65Dn mice. Here, we concentrated on short-term memory impairment. In this case the cortical regions are essential to the temporary storage and the recall of information over short time periods, a general process known as working memory. Lesion experiments have shown that the prelimbic area is critically involved in working memory (Granon et al., 1994). Working memory is impaired in DS (Lanfranchi et al., 2014). Ts65Dn and Ts1Rhr models of DS with partial trisomy of Mmu16 that includes the *Dyrk1a* gene (Belichenko et al., 2009; Faizi et al., 2011) have impaired short-term memory in the spontaneous alternation paradigm. Normalization of the *Dyrk1a* copy number in TS65Dn mice improves working memory (Garcia-Cerro et al., 2014), indicating that overexpression of DYRK1A is involved in working memory alterations. In a single-gene model like the mBACtgDyrk1a mouse, our results are consistent with the idea that mice overexpressing DYRK1A have an impaired working memory. Use of EGCG treatment either in POL60 or in decaffeinated MGTE rescued working memory in a Y-maze paradigm. MGTE treatment was assessed in a pilot human clinical trial and reversed the working memory deficit in individuals with DS (De la Torre et al., 2014). Our results on the levels of synaptic markers suggest that this rescue is linked with the effect of EGCG on E/I balance. However, molecular analyses indicate that POL60 treatment induces a stronger correction of the level of proteins involved in the GABAergic pathway than decaffeinated MGTE, an effect that appears to be mediated, in part, by the presence of caffeine in POL60. We have recently shown that inhibition of DYRK1A is acting on GABA-producing enzymes at two different levels, by controlling levels of GAD67 or GAD65 proteins, but also by controlling the activity of these enzymes: the level of pyridoxal phosphate, a coenzyme of GAD67 and GAD65, is under the control of DYRK1A (Tlili et al., 2013): therefore an EGCG treatment can modify the activity of GADs enzymes by inhibiting DYRK1A activity.

Conclusion

Results show that EGCG treatment of adult mice reverses brain molecular alterations that disrupt E/I balance. Two different extracts are also efficient to restore working memory in a single-gene model and in a partial trisomy model of DS. DYRK1A is thus a therapeutic target for Down syndrome. The panel of proteins involved in the control of synaptic plasticity and E/I balance is potentially useful to assess consequences of other therapeutic strategies, and its use may help to understand molecular mechanisms involved in these strategies. The partial rescue of components of the GABAergic pathway observed when treating adult mice with a decaffeinated green tea extract suggest the possibility of combining two drugs such as EGCG and an inverse GABA agonist (Braudeau et al., 2011) to reach a complete rescue of GABAergic and glutamatergic pathways.

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Supplementary Material

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frontiers in Behavioral Neuroscience

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Principal Component Analysis of the Effects of Environmental Enrichment and (-)-epigallocatechin-3-gallate on Age-Associated Learning Deficits in a Mouse Model of Down Syndrome

Silvina Catuara-Solarz[1,2†], Jose Espinosa-Carrasco[1,2,3†], Ionas Erb[2,3†], Klaus Langohr[4,5], Cedric Notredame[2,3], Juan R. Gonzalez[3,6,7] and Mara Dierssen[1,2,8*]*

[1] Systems Biology Program, Cellular and Systems Neurobiology, Centre for Genomic Regulation, The Barcelona Institute of Science and Technology, Barcelona, Spain, [2] Centre for Genomic Regulation, Universitat Pompeu Fabra, Barcelona, Spain, [3] Bioinformatics and Genomics Program, Comparative Bioinformatics, Centre for Genomic Regulation, Barcelona Institute of Science and Technology, Barcelona, Spain, [4] Neurosciences Research Program, Human Pharmacology and Clinical Neurosciences Research Group, IMIM (Hospital del Mar Medical Research Institute), Barcelona, Spain, [5] Department of Statistics and Operations Research, Universitat Politècnica de Catalunya/BARCELONATECH, Barcelona, Spain, [6] Centre for Research in Environmental Epidemiology, Barcelona, Spain, [7] Centro de Investigación Biomédica en Red de Epidemiología y Salud Pública, Barcelona, Spain, [8] Centro de Investigación Biomédica en Red de Enfermedades Raras, Barcelona, Spain

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***Correspondence:**
Juan R. Gonzalez
jrgonzalez@creal.cat;
Mara Dierssen
mara.dierssen@crg.eu

† These authors have contributed equally to this work.

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Down syndrome (DS) individuals present increased risk for Alzheimer's disease (AD) neuropathology and AD-type dementia. Here, we investigated the use of green tea extracts containing (-)-epigallocatechin-3-gallate (EGCG), as co-adjuvant to enhance the effects of environmental enrichment (EE) in Ts65Dn mice, a segmental trisomy model of DS that partially mimics DS/AD pathology, at the age of initiation of cognitive decline. Classical repeated measures ANOVA showed that combined EE-EGCG treatment was more efficient than EE or EGCG alone to improve specific spatial learning related variables. Using principal component analysis (PCA) we found that several spatial learning parameters contributed similarly to a first PC and explained a large proportion of the variance among groups, thus representing a composite learning measure. This PC1 revealed that EGCG or EE alone had no significant effect. However, combined EE-EGCG significantly ameliorated learning alterations of middle age Ts65Dn mice. Interestingly, PCA revealed an increased variability along learning sessions with good and poor learners in Ts65Dn, and this stratification did not disappear upon treatments. Our results suggest that combining EE and EGCG represents a viable therapeutic approach for amelioration of age-related cognitive decline in DS, although its efficacy may vary across individuals.

Keywords: Down syndrome, aging, (-)-epigallocatechin-3-gallate, Morris water maze, principal component analysis

INTRODUCTION

Down syndrome (DS) is the most prevalent genetic cause of intellectual disability arising from trisomy of chromosome 21 with an incidence of approximately 1 in 1000 live births worldwide. DS affects the development and function of the central nervous system throughout life, leading to a distinctive profile of cognitive impairment and increased risk for Alzheimer's disease (AD)

neuropathology. By the age of 40, almost all DS adults develop AD-like neuropathology and by the age of 55–60, around 70% develop dementia (Wilcock and Griffin, 2013). DS brains exhibit extracellular deposition of amyloid-ß (Aβ), following a fronto-striatal pattern (Wisniewski et al., 1985; Mann, 1988; Lemere et al., 1996), while hyperphosphorylated tau, in the form of neurofibrillary tangles, accumulates later in life affecting mainly the hippocampal formation, the entorhinal cortex, and the neocortex (Hof, 1995; Hyman, 1995).

So far, therapeutic interventions aimed at slowing down cognitive decline in AD such as N-methyl-D-aspartate (NMDA) receptor antagonists (memantine), anticholinesterase inhibitors (donepezil, rivastigmine, galantamine), or GABA-A antagonists have not been able to demonstrate improvements in cognitive performance in demented nor in young non-demented DS subjects (De la Torre and Dierssen, 2012). In recent years, treatment with (-)-epigallocatechin-3-gallate (EGCG), the most abundant polyphenol found in green tea, has gained attention as it has beneficial effects in AD mouse models possibly contributed by its antioxidant activity, free radical scavenging, iron chelating, anti-inflammatory effects, neuroprotection, and promotion of the non-amyloidogenic pathway of APP through ADAM10 maturation (Obregon et al., 2006; Kalfon et al., 2007; Rezai-Zadeh et al., 2008; Biasibetti et al., 2013; Kim et al., 2014). Interestingly, EGCG also has inhibitory properties on the kinase activity of DYRK1A (Bain et al., 2003; Adayev et al., 2006; Wang et al., 2012), a DS candidate whose overabundance is associated with DS neurocognitive symptoms and neurodegenerative phenotypes (Becker et al., 2014). EGCG ameliorates cognitive deficits not only in AD and DS mouse models, but also in young adults with DS (Lee et al., 2013; De la Torre et al., 2014).

Additionally, non-pharmacological therapeutic intervention, such as environmental enrichment (EE), has been successfully used in mouse models of AD (Jankowsky et al., 2005; Lazarov et al., 2005; Berardi et al., 2007; Li et al., 2013; Polito et al., 2014) and DS (Martínez-Cué et al., 2002, 2005; De la Torre et al., 2014). Interestingly, many of the effects reported for EE are similar to those observed upon EGCG treatment, such as neuroplasticity enhancement, antioxidant activity, anti-inflammatory function, neuroprotection, and promotion of the non-amyloidogenic proteolytic pathway of APP (Ickes et al., 2000; Jankowsky et al., 2005; Birch et al., 2013; Mármol et al., 2015). In fact, EE has also been shown to normalize the expression levels and the kinase activity of DYRK1A in mice overexpressing Dyrk1A and in Ts65Dn mice (Golabek et al., 2011; Pons-Espinal et al., 2013).

In the present study, we investigated the effects of combined treatment with EGCG and EE on hippocampal-dependent learning and memory, which is one of the cognitive domains most susceptible to age-associated decline and primarily affected in AD and DS (Granholm et al., 2000). To this end, we used the Ts65Dn mouse model of DS, which bears a segmental trisomy for MMU16 (syntenic region to HSA21) from Mrpl39 to Zfp295 covering APP and DYRK1A, and shows predictive validity with DS (reviewed in Dierssen, 2012) including AD-like cholinergic neuronal loss and age-associated cognitive decline (Holtzman et al., 1996; Seo and Isacson, 2005; Contestabile et al., 2006). The Ts65Dn mouse model only partially recapitulates AD pathology since it does not exhibit extracellular β-amyloid-containing plaques or neurofibrillary tangles. However, it develops other abnormal neuronal processes associated to Aβ production such as enlarged neuronal early endosomes, or increased immunoreactivity for markers of endosome fusion and recycling (Cataldo et al., 2003) that lead to alterations in NGF retrograde transport from the hippocampus to the BF (Salehi et al., 2006). Thus, it is an adequate model to investigate potential therapeutic interventions to tackle some of the common pathogenic mechanisms between DS and AD.

We assessed the effects of the treatments on spatial learning and memory performance in the Morris water maze by using classical single-variate measures such as escape latency, Gallagher index or thigmotaxis. However, learning is a process that involves the orchestration of a myriad of cognitive and behavioral outcomes, and a single variable cannot capture its essence. Learning is also measured by variables that are themselves influenced by different factors. Only under certain conditions will these measures provide the information they were designed for (e.g., latency is a good measure if all animals have the same speed, or the time spent in the periphery if it is associated with thigmotactic behavior). Such idealizations are hard to justify in an experimental context where high variability between subjects is the rule, not the exception. PCA allowed to assess the learning impairment in Ts65Dn mice and the effects of EE, EGCG, and EE-EGCG treatments in a less variable-dependent manner. We examined the relative contribution of seven behavioral variables to the variance in the data obtained from multiple water maze measurements. We identified two composite variables that together explained 86% of the variance among groups: one related to learning, and the other one mainly measuring the component of swimming speed that is not target-directed.

MATERIALS AND METHODS

Ts65Dn Mouse Colony

Ts65Dn and wild type (WT) littermate mice were obtained through repeated crossings of B6EiC3Sn a/A-Ts(17^{16})65Dn (Ts65Dn) females to B6C3F1/J males purchased from The Jackson Laboratory (Bar Harbor, ME). The mouse colony was bred in the Animal Facilities of the Barcelona Biomedical Research Park (PRBB, Barcelona, Spain, EU). Mice were housed in standard or enriched conditions (see below) under a 12:12 h light–dark schedule (lights on at 8:00 a.m.) in controlled environmental conditions of humidity (60%) and temperature (22 ± 2°C) with food and water *ad libitum*. Both the Ts65Dn and euploid mice were genotyped by qPCR, in accordance with the Jackson laboratories protocol (https://www.jax.org/research-and-faculty/tools/cytogenetic-and-down-syndrome-models-resource/protocols/cytogenic-qpcr-protocol).

Experiments were conducted using 5–6 months old female mice. This age represents the starting point of gradual cognitive decline (Granholm et al., 2000) and we used females since Ts65Dn males show high levels of stress in EE conditions that could mask the effect of the treatments (Martínez-Cué et al., 2002). All animal procedures met the guidelines of European

Community Directive 2010/63/EU and the local guidelines (Real Decreto 53/2013) and were approved by the Local Ethics Committee (Comité Ético de Experimentación Animal del PRBB (CEEA-PRBB); procedure numbers MDS-08-1060P2 and MDS-14-1611).

Treatment: Environmental Enrichment Housing Conditions and (-)-epigallocatechin-3-gallate (EGCG)

Ts65Dn and WT 5–6 months old female mice were randomly assigned to one of the following experimental groups: no treatment (NT), environmental enrichment (EE), green tea extract containing 45% (-)-epigallocatechin-3-gallate (EGCG), or a combination of EE and EGCG (EE-EGCG). Mice received the different treatments for 30 days based on previous studies (Pons-Espinal et al., 2013; De la Torre et al., 2014). In the NT condition animals were reared in conventional cages (20 × 12× 12 cm height, Plexiglas cage) in groups of 2–3 animals. EE housing consisted of spacious (55 × 80× 50 cm height) Plexiglas cages with toys, small houses, tunnels, and platforms of different shapes, sizes, colors and textures. Wheels were not introduced in the cages in order to avoid the effect of physical exercise. The arrangement was changed every 2 days to keep novelty conditions. To stimulate social interactions, 6–8 mice were housed in each cage. EGCG was administered in drinking water (EGCG dosage: 0.326 mg/ml, 0.9 mg per day; 30 mg/Kg per day) by preparing fresh EGCG solution every 2 days from a green tea leaf extract [Mega Green Tea Extract, Decaffeinated, Life Extension®, USA; EGCG content of 326.25 mg per capsule]. Even if there were fluctuations in EGCG dosage due to drinking volume and mice weight there were no significant differences in mean EGCG intake along days between WT (29.79 mg/Kg per day) and Ts65Dn (32.59 mg/Kg per day) mice (data not shown). The sample size for each experimental group was the following: WT = 10; TS = 11; WT-EE = 14; TS-EE = 11; WT-EGCG = 11; TS-EGCG = 9; WT-EE-EGCG = 12; TS-EE-EGCG = 8.

Morris Water Maze

The water maze consisted of a circular pool (1.70 m diameter; 0.6 m height) filled with tepid water (19 ± 2°C) opacified by the addition of white non-toxic paint. White curtains with affixed black patterns surrounded the maze to provide an arrangement of spatial cues. The settings enabled a spatial allocentric learning and memory task based on distal cues (Vorhees and Williams, 2006). The first day mice were habituated to the task at the pre-training session in which the escape platform (12 cm diameter, height 24 cm) was located at the center of the pool and was visible by 1 cm over the water level. During the following 5 days mice learned the position of the platform, which was hidden 1 cm below water (northeast quadrant, 22 cm away from the wall) in 4 training (acquisition) trials per day. In each trial, mice were placed at one of the starting locations in random order (north, south, east, west), including permutations of the four starting points per session, and were allowed to swim until they located the platform. Mice failing to find the platform within 60 s were placed on it for 20 s and were returned to their home cage at the end of every trial. To assess the reference memory a probe session was performed 24 h after the last acquisition session. The platform was removed and mice were allowed to swim for 60 s during which the % of time spent in the target quadrant and the proximity to platform (Gallagher index) was calculated by sampling the position of the animal in the maze (10 times per second) to provide a record of its distance to the escape platform in 1-s averages (Gallagher et al., 1993). The cued session was performed to test the mice motivation to find the platform and visual ability using the platform elevated 1 cm above the water with its position clearly indicated by a visible cue (black flag). Mice that did not reach the platform in less than 30 s in this session were considered unsuitable for the test and were subtracted from the analysis. During days 8–10, cognitive flexibility, the ability of mice to re-learn a new location of the platform, was assessed in the reversal sessions in which the platform was located at the opposite quadrant. There was 1 missing subject on the reversal sessions.

All the trials were recorded with an image tracking system (SMART, Panlab, Spain) connected to a video camera placed above the pool. Escape latencies, length of the swimming trajectories and swimming speed for each animal and trial were monitored and computed. The analysis of mice performance was conducted using a custom-designed analysis program, Jtracks software, which generates heat-maps of the spatial distribution of the accumulated trajectories in each group. Jtracks was further used to obtain other measurements such as the Gallagher index and the Whishaw index, defined as the percentage of path inside the optimal corridor connecting release site and goal, to quantify the most efficient and direct trajectory from the location of mice to the platform (Whishaw and Jarrard, 1996).

Statistical Analysis

Two questions were addressed: the global differences over time and the progression of learning across sessions. The first question was tested by single variate analysis of the differences between experimental groups for three learning-related parameters (latency to reach the platform, Gallagher index and % of time spent in the periphery). Data were expressed as mean + S.E.M and analyzed using One-way ANOVA or ANOVA repeated measures. The second question was evaluated by estimating the linear effect of time-group interaction using a general linear-mixed model for each behavioral parameter. We associated random-effects terms with the animal factor in order to model within-subject correlation that appears due to the repeated nature of the data. Also, the variable "latency" was right-censored, since mice are allowed to swim a maximum of 60 s (Vock et al., 2012). Estimation of the coefficients and their associated *p*-values were based on maximum log-likelihood methods using the R library censReg (Henningsen, 2013). We used the plot of the model residuals vs. the fitted values to check model assumptions. Multiple comparisons for parametric model were used to address *post-hoc* comparisons using multest R package and glht function (Hand and Taylor, 1987; Dickhaus, 2012). Non-treated WT and Ts65Dn were considered as the reference groups for the comparisons. To control the false discovery rate (FDR) due to multiple *post-hoc* comparisons Benjamini-Hochberg method

was used (Benjamini and Hochberg, 1995). This procedure was implemented both for the ANOVA and for the linear-mixed model in the R package multtest (Pollard et al., 2005).

Principal Component Analysis

The "learning" process is composed by many variables whose influence on performance may be great for some, whereas for others it may be so small that they can be ignored. For example, you might start with ten original variables, but might end with only two or three meaningful axes. This is known as reducing the dimensionality of a data set. PCA is the most commonly used technique to identify linear combinations of variables in a high-dimensional space best representing the variance that is present in the data. This is achieved by considering each variable to be an axis in a high-dimensional space. Individuals, or groups of individuals, can be represented as points in this space. PCA identifies a linear combination of the original variables, called principal component that accounts for the largest amount of the experimental variability. Once this first principal component is set, PCA finds successive orthogonal principal components that explain the maximum amount of the remaining variance given that the orthogonality constraint is met. Finally, the original data and the original variables can be projected in this new space defined by the principal components. In our analysis we were mainly interested in the variation among experimental groups as well as the variation of a given group along the learning sessions. To find the variables best representing these two types of between-group variation (within- and between-learning sessions), we used the group medians of each variable on each acquisition day. A supervised analysis using group means instead of variables measured on individuals is known as discriminant analysis, (c.f. Greenacre, 2010). Such methods are suitable for the analysis of behavioral data having several conditions with a number of replicates per condition. For reasons of robustness to outliers, however, we here prefer to use the medians instead of the means. The PCA was performed on 40 observations (eight experimental groups on five learning sessions, where the four trials of each learning session were averaged) corresponding to median group performances of seven variables on each acquisition day. Separately, a similar analysis was done for the three reversal sessions.

The variables of interest were latency to target, percentage of time spent in target quadrant, percentage of time spent in the periphery, Whishaw index, Gallagher index, distance traveled, and speed. To allow for the combination of the original variables measured in different units, all variables were scaled to unit variance before the analysis (the default Z-score scaling was used).

Since the PCA was performed on group medians (grouped data), points identified in the PCA space will correspond to groups of individuals. To identify points corresponding to individuals themselves, we used the technique of "adding supplementary points." Given a single measurement corresponding to a point in the space of the original variables, we can identify the new coordinates of this point in the space defined by the principal components. Note that such points will not change the coordinate system, as they are added after the PCA is performed. Adding all 86 individuals appearing five times each as supplementary points, we identified the coordinates for each of the individuals. The R-package FactoMineR (Lê et al., 2008) was used for the PCA as it allowed for a straightforward inclusion of supplementary observations. Density plots were obtained using the statdensity_2d function from the ggplot2 R package (Wickham, 2009) with the parameters: $n = 100$, $h = 5$, and bins $= 6$.

Permutation Test

To assess statistical significance of group separation, we performed a permutation test, a standard procedure in multivariate data analysis (Sham and Purcell, 2014). Individuals were drawn and reassigned randomly to experimental groups. Correct acquisition sessions were maintained, and thus each individual kept their learning performance along acquisition (i.e., all five values corresponding to the learning sessions of an individual were assigned to the same group). Group medians were then determined for each learning session for these new groups. Original numbers of individuals in each group were kept. To determine overall group separation, percentage of within-session variance (see variance decomposition below) was used as a statistic. For learning differences, we used a t-statistic involving PC1 pairwise group comparisons. All pairwise comparisons were determined at each permutation. The number of randomized PCAs was 10,000.

Variance Decomposition

Total, between-group, between-session, and within-session variances were directly calculated from the (standard) coordinates obtained from the PCA. Variance in the PCA was calculated from the distances d of objects i from the origin:

$$V = \frac{1}{7N}\sum_{i=1}^{N} d_i^2$$

where the factor 1/7 comes from the number of variables. In the case of between-group variance V_B, the objects i are the groups and $N = 40$. Since we performed the PCA on the groups, by construction the between group variance sums to 1. For the total variance V_T, the objects are the individuals (supplementary points) and $N = 430$. The percentage of between-group variance is then V_B/V_T x 100. The usual definition of V_B is for the group averages, not medians, which means that here we are actually estimating a lower bound on the percentage of between-group variance. To obtain the between-sessions variance V_{BS}, we calculated the squared mean distance from the origin over all groups on a given acquisition session s:

$$d_s^2 = \sum_{p=1}^{7}\left(\frac{1}{8}\sum_{j=1}^{8} x_{s,p,j}\right)^2$$

where the $x_{s,p,j}$ are the standard coordinates for principal axis p of an experimental group j during a given session s. Then we used the first formula for the variance with $N = 5$. Within-session variance is the difference $V_B - V_{BS}$ (which can again be expressed as a percentage of total variance V_T).

RESULTS

To evaluate the functional impact of EE, EGCG, and the potential synergistic effects of a combination of EE-EGCG on the age-associated hippocampal-dependent learning and memory deficits of Ts65Dn mice, we compared the behavioral performance of mice treated with EE, EGCG, or EE-EGCG in the MWM with their untreated controls of both WT and Ts65Dn genotypes.

Two different questions were addressed: the overall learning differences among the experimental groups on single learning variables (escape latency, Gallagher index etc.) and the effects of treatments on the progression of learning across sessions (slope of the learning curve). The first question was evaluated by analyzing the effect of the group variable (defined by genotype and treatment) by one-way repeated measures ANOVA. The second question was evaluated by estimating the linear effect of time-group interaction with a general linear-mixed model on each behavioral parameter, censored for latency.

Effects of EE, EGCG, and EE-EGCG Treatments

First we performed a classical single-variate analysis including relevant parameters for the learning process. During the habituation (pre-training) session, all groups behaved in a similar manner, with no differences in the latency to reach the visible platform [overall genotype-treatment effect $F_{(7,\ 78)} = 0.937$; *p*-value *n.s.*] or the mean distance to the platform, as quantified by the Gallagher index [overall genotype-treatment effect $F_{(7,78)} = 1.161$; *p*-value *n.s.*] indicating no genotype- or treatment-dependent differences in procedural learning (**Figure 1A**).

Along the acquisition sessions, untreated WT mice efficiently learned the platform position, as shown by the progressive reduction in the latency to reach the hidden platform and the increasing preference for the target quadrant (**Figures 1A–C**). As it has been previously reported, we detected impaired learning ability in untreated Ts65Dn mice, shown by the higher latency to reach the hidden platform across days that was not reduced

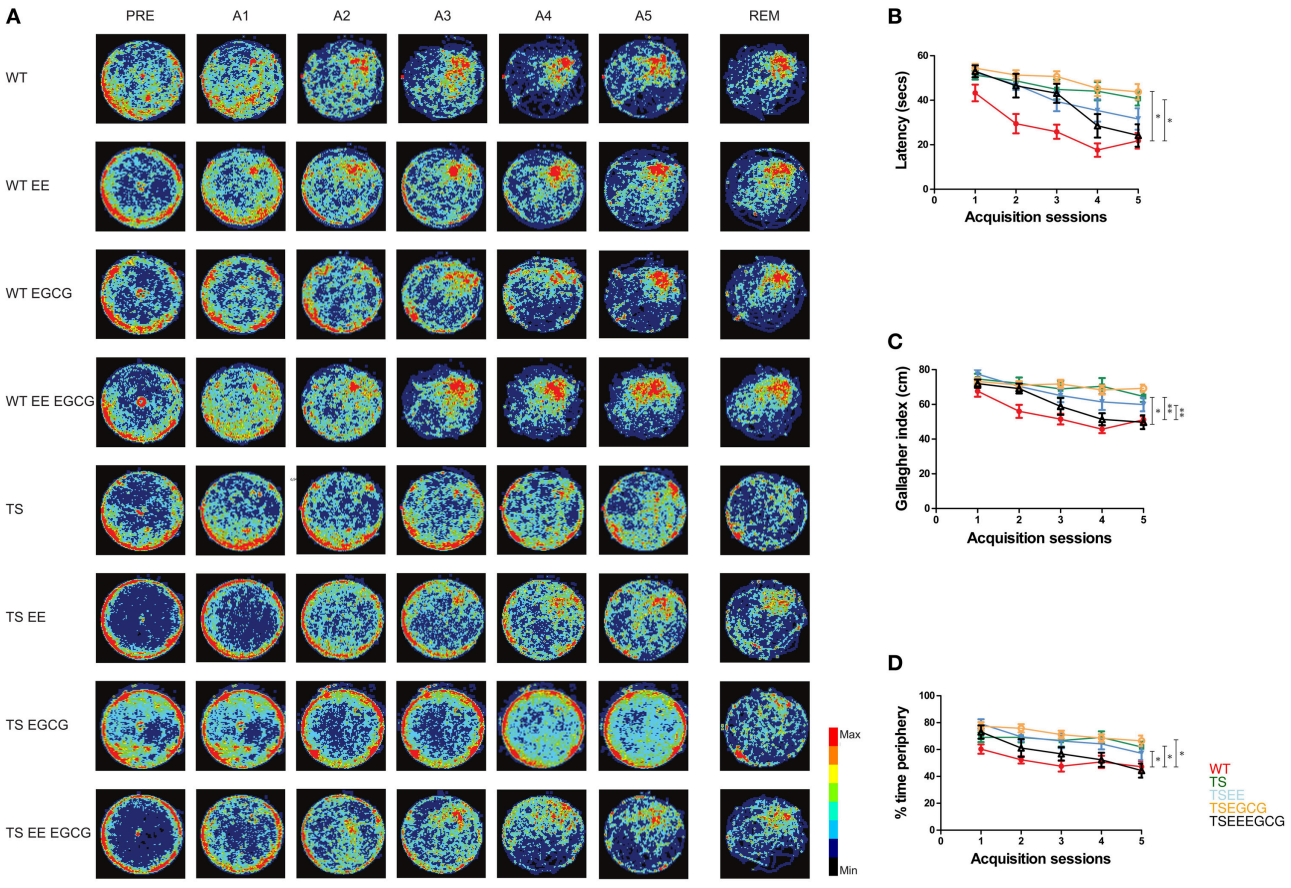

FIGURE 1 | EE-EGCG treatment is more efficient than EE or EGCG alone to ameliorate the hippocampal-dependent learning and memory alterations of middle age Ts65Dn mice. (A) Heat-map representing the accumulated trajectories of mice from the different experimental groups [untreated wild type (WT) = 10, untreated Ts65Dn (TS) = 11 WT-EE = 14; TS-EE = 11; WT-EGCG = 11; TS-EGCG = 9; WT EE-EGCG = 12; TS EE-EGCG = 8] across sessions in the Morris water maze. Color scale is depicted on the right, where red corresponds to the most visited zones and black to the less or non-visited zones. **(B)** Latency (seconds to reach the escape platform). **(C)** Gallagher index (mean distance between subject and goal in cm). **(D)** Thigmotaxis (percentage of time spent on the periphery). Data in **(B–D)** are represented as mean ± SEM. Data were analyzed with ANOVA repeated measures with Tukey *post-hoc* comparisons corrected with BH; $^{*}p < 0.05$, $^{**}p < 0.01$. PRE, pre-training; A1-5, acquisition days 1–5 with 4 trials per day; REM, removal. All the possible *post-hoc* comparisons were performed but only treated TS, and untreated WT and TS groups are shown in the figure.

across sessions, leading to a flatter learning curve ($\beta = -3.05$; p-value < 0.01, **Figure 2A**) in comparison to untreated WT. Trisomic mice also showed increased global Gallagher index [overall genotype-treatment effect $F_{(7, 78)} = 7.072$, p-value < 0.01; Tukey *post-hoc* BH corrected p-value < 0.01, **Figure 1C**] and the typical increased thigmotaxis [higher percentage of time spent close to the pool periphery; overall genotype-treatment effect $F_{(7, 78)} = 6.12$, p-value < 0.01; Tukey *post-hoc* BH corrected p-value < 0.05, **Figure 1D**] that has been previously reported (Reeves et al., 1995).

EE ameliorated the deficits found in Ts65Dn mice as shown by a reduction of the escape latency across acquisition sessions as compared to untreated trisomic mice ($\beta = -2.92$; p-value < 0.05, **Figure 2A**). Interestingly, enriched Ts65Dn (TS-EE) mice also exhibited a more goal-directed behavior as shown by a progressive reduction of the Gallagher index ($\beta = -2.23$; p-value < 0.05, **Figure 2B**) and of thigmotactic behavior ($\beta = -3.47$; p-value < 0.05, **Figures 1A**, **2C**) in comparison to untreated trisomic mice. However, Ts65Dn mice under EE still showed poorer performance when compared to WT as reflected by higher Gallagher index values (Tukey *post-hoc* BH corrected p-value < 0.01, **Figure 1C**) and thigmotaxis (Tukey *post-hoc* BH corrected p-value < 0.05, **Figure 1D**).

Conversely, Ts65Dn mice treated with EGCG (TS-EGCG) did not show any effects of treatment. In this group, neither the latency to reach the platform ($\beta = -1.99$; p-value = *n.s.*, **Figure 2A**) nor the Gallagher index ($\beta = -2.09$; p-value = *n.s.*, **Figure 2B**), were improved as compared to untreated Ts65Dn mice. In fact, TS-EGCG mice exhibited increased thigmotactic behavior ($\beta = -5.39$; p-value < 0.01, **Figure 2C**).

Finally, the combined treatment with EE-EGCG significantly improved performance in Ts65Dn mice, markedly reducing the latency to reach the platform ($\beta = -4.83$; p-value < 0.01, **Figure 2A**), Gallagher index ($\beta = -4.04$; p-value < 0.01, **Figure 2B**) and thigmotaxis ($\beta = -5.18$; p-value < 0.01, **Figure 2C**) across the acquisition sessions as compared to untreated trisomic mice. In fact, the combined treatment effects in Ts65Dn mice reached values that were not statistically different from those of untreated WT mice in latency ($\beta = -1.78$; p-value = *n.s.*, **Figure 2A**), Gallagher index (Tukey *post-hoc* BH corrected p-value = *n.s.*, **Figure 1C**) and thigmotaxis (Tukey *post-hoc* BH corrected p-value *n.s.*, **Figure 1D**) suggesting a rescue of the phenotype.

Neither of the treatments had effects on the latency to reach the platform, nor on the Gallagher index in WT mice. However, both EE ($\beta = -3.11$; p-value < 0.05, Figure S1) and EE-EGCG ($\beta = -3.94$; p-value < 0.01, Figure S1) promoted a significant reduction in the percentage of time in the periphery along acquisition days.

There were no differences in swimming speed between untreated WT and Ts65Dn mice (overall gen-treatment effect $F_{(7, 8)} = 2.820$; p-value < 0.05; Tukey *post-hoc* comparisons corrected by BH showed p-value = *n.s.*; data not shown). On the other hand EGCG treatment had a significant effect reducing swimming speed on WT (Tukey *post-hoc* BH corrected p-value < 0.05; data not shown) and Ts65Dn (Tukey *post-hoc* BH corrected p-value < 0.01; data not shown) in comparison with untreated WT. The rest of the treatments showed no effect on swimming speed during learning.

Effects of EE, EGCG, and EE-EGCG Treatments on Reference Memory and Cognitive Flexibility

To assess the reference memory a probe trial was performed 24 h after the last acquisition day. The percentage of time spent in the target quadrant showed a tendency in untreated Ts65Dn to perform worse than WT and also in EE-EGCG treated Ts65Dn mice to perform better than untreated Ts65Dn, however there were no statistically significant differences among the groups [overall genotype-treatment effect $F_{(7, 78)} = 1.498$; p-value = *n.s.*; Figure S2]. This was probably due to the large within-group variance as depicted in the boxplots, by the large

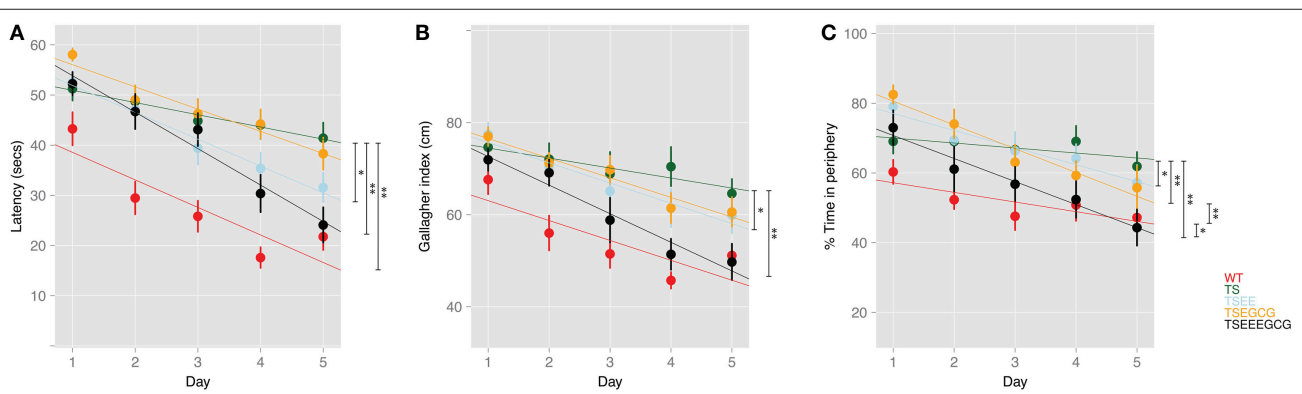

FIGURE 2 | Linear mixed model reveals improvement of hippocampal-dependent learning upon EE and EE-EGCG treatment in middle age Ts65Dn mice. Fitted linear mixed model (represented as colored lines) and observations (dots) represented as mean ± SEM of **(A)** Latency to reach the escape platform (log-transformed censored model), **(B)** Gallagher index, and **(C)** thigmotaxis along learning sessions. The model enabled the comparison of the slope of the learning trajectory (β) across days among experimental groups. *Post-hoc* comparisons were corrected with BH; *$p < 0.05$, **$p < 0.01$; non-treated WT and TS were considered as references for the comparisons.

distance between the box edges (25th and 75th percentiles; Figure S2). On the other hand, the Gallagher index, which is a more precise measure, presented less within-group variance and showed global differences in performance among experimental groups [overall genotype-treatment effect $F_{(7, 78)} = 2.741$; p-value < 0.05, **Figure 3**]. Tukey *post-hoc* comparisons adjusted by the BH method showed that untreated Ts65Dn presented higher Gallagher index than untreated WT mice (p-value < 0.05) indicating poor reference memory. The administration of EGCG (Tukey *post-hoc* BH corrected p-value = *n.s*) or EE (Tukey *post-hoc* BH corrected p-value = *n.s*) alone did not affect the Ts65Dn reference memory deficit. However, the combination of EE-EGCG reduced the Gallagher index in Ts65Dn (Tukey *post-hoc* BH corrected, p-value = 0.05), reaching a performance that was similar to WT (Tukey *post-hoc* BH corrected p-value = *n.s.*, **Figure 3**).

Cognitive flexibility was assessed along the reversal sessions. While untreated WT mice clearly shifted their search to the new platform location, untreated Ts65Dn mice persevered searching the old platform location. This poorer cognitive flexibility was reflected in an increased latency to reach the new platform position [overall genotype-treatment effect $F_{(7, 77)} = 5.648$, p-value < 0.01; Tukey *post-hoc* BH corrected p-value < 0.01, Figure S3A], a trend toward an increased Gallagher index [overall genotype-treatment effect $F_{(7, 77)} = 7.438$; Tukey *post-hoc* BH corrected p-value = 0.06, Figure S3B] and increased thigmotaxis across the 3 reversal learning sessions [overall genotype-treatment effect $F_{(7, 77)} = 4.570$; Tukey *post-hoc* BH corrected p-value < 0.05; Figure S3C], as compared to WT. Even though there were no significant effects of any of the treatments on Ts65Dn latency to reach the new platform positions, both TS-EE ($\beta = 11.78$, p-value < 0.05, Figure S3A) and TS-EE-EGCG ($\beta = 11.95$, p-value < 0.05, Figure S3A) were qualitatively less different from WT than untreated Ts65Dn mice ($\beta = 18.46$, p-value < 0.01, Figure S3A) taking into account the magnitude of the group differences by the model estimate (β). Neither of the treatments had effects on the Gallagher index (Figure S3B) nor the thigmotaxis on Ts65Dn or WT mice during the reversal sessions (Figure S3C).

Multidimensional Analysis of Learning Impairment in Ts65Dn Mice: Global Effects of EE, EGCG, and EE-EGCG Treatments

There is not a single best measure of learning (such as the classical "escape latency" or "distance traveled") and thus, discrimination of learning performance differences could be better achieved by a combination of some of these variables. Thus, to go a step further, we used permutation-validated principal component analysis (PCA), to determine which combination of the experimental variables would be best suited to describe the differences in learning among our groups. Significance of differences was determined by permutation-based test statistics.

Variables related to the learning improvement along the five learning sessions, including Gallagher index, % time spent in target quadrant, distance traveled, percentage of time spent in periphery, Whishaw index and latency to target, loaded on PC1, which accounted for 74% of the (between-group) variance (**Figures 4A,C**). High values of PC1 correspond to short distances to target, low latencies, high percentages of time in the target quadrant, etc. (**Figure 4B**). This axis can be understood as a new composite learning measure. In contrast, the second principal axis (PC2, 12% of variance) is dominated by the contribution of swimming speed and thus is mainly dependent on motor ability. By construction, it is independent from the learning-related PC1. It is noteworthy that speed also contributes

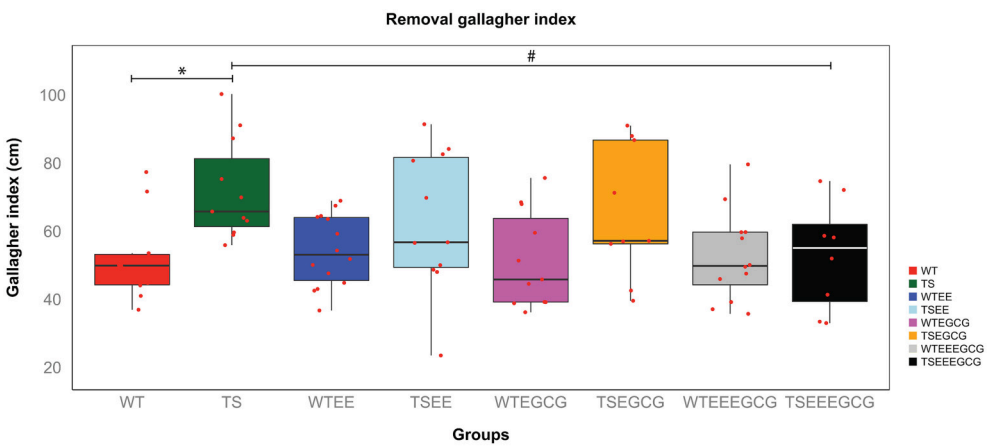

FIGURE 3 | EE-EGCG treatment is more efficient than EE or EGCG alone to ameliorate reference memory at the probe trial in Ts65Dn group. The figure shows boxplots of the distribution of the distance to the target (Gallagher index) of all experimental groups in the removal session. In each boxplot, the horizontal line corresponds to group median, the box edges gives the 25th and 75th percentiles and the whiskers depict minimum and maximum values to a maximum of 1.5 times the interquartile distance from the box, and more extreme values are individually plotted. Red dots indicate the values of each individual mouse. TS-EE-EGCG showed a reduction of Gallagher index when compared to untreated TS mice that is not observed in the rest of TS mice groups. ANOVA, with Tukey *post-hoc* comparisons corrected with BH; $^{*}p < 0.05$; $^{\#}p = 0.05$; comparisons were performed to test the differences between genotypes and the effects of treatments on the TS.

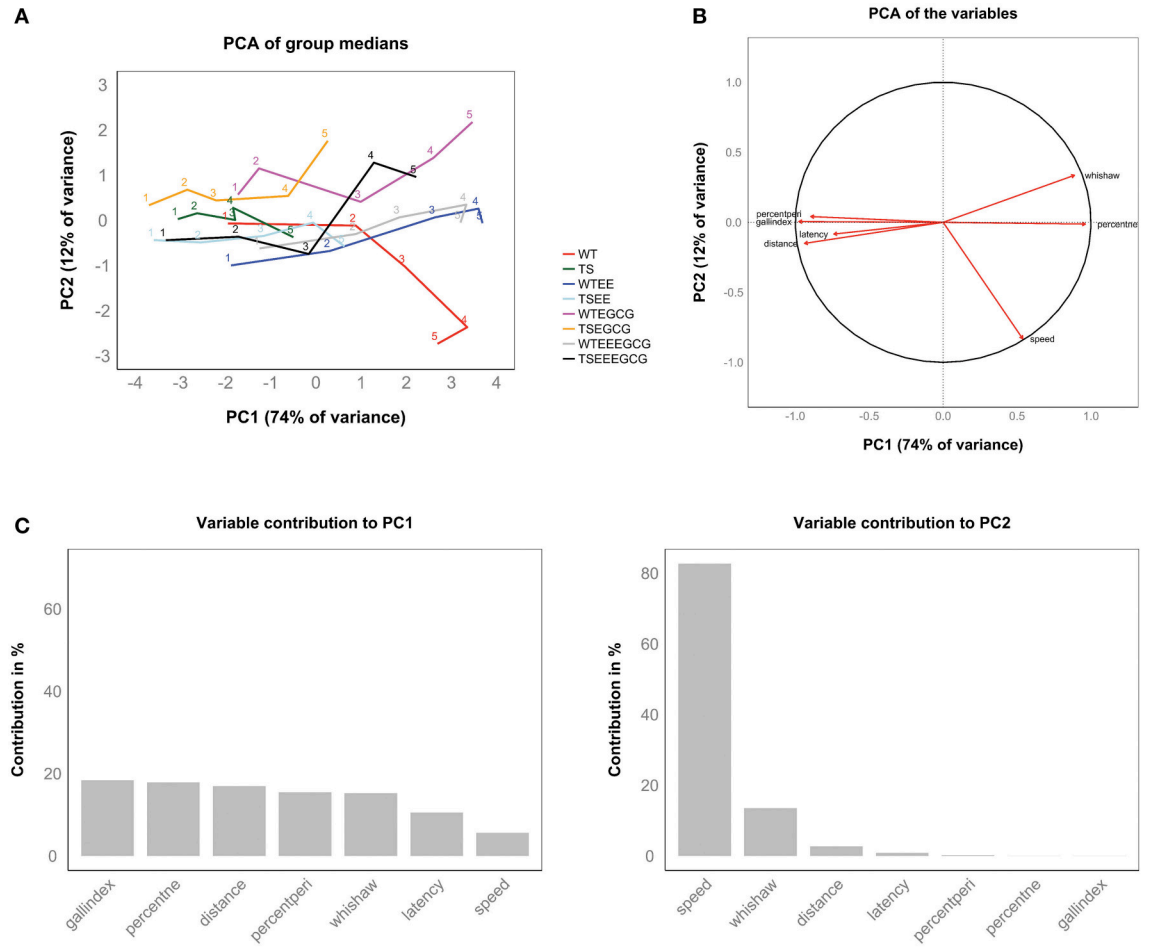

FIGURE 4 | Supervised PCA of the experimental groups during the acquisition sessions revealed the main direction of learning along the first principal component. (A) Distribution of the group performance (medians) in the new ordination space, which consists of linear combinations of the original variable space. Each trajectory represents an experimental group and connects the five learning sessions labeled with its respective number. All group trajectories showed a progression toward positive values of the first principal component (PC1). For a given learning session, experimental groups achieving better performance attain higher values on this axis. The progression of trajectories on the second principal component (PC2) appears more erratic. **(B)** PCA of the variables, where arrows represent the direction of each variable in the PCA space. Arrows reaching the unit circle belong to variables that are well represented by the two principal components. **(C)** Bar plots showing the percentage of explained variance for each principal component. Bars represent the contribution (%) of each variable to first and second principal components. The first principal component (left panel) can be interpreted as a composite learning variable where classical variables used to assess learning had major and similar contribution ranging from 18% in the case of the Gallagher index to 10% in the case of the latency. Speed (right panel) constitutes the main contributor to PC2 (82%), but is split between PC1 and PC2 in almost equal parts (see panel **B**).

to PC1, where it shows a relation to learning (animals that have learned the target position tend to go there faster). Speed is thus decomposed in a learning-dependent component and a learning-independent component more related with the intrinsic motor capability of mice (**Figure 4B**).

Each of the eight experimental groups is represented as a trajectory connecting five dots that correspond to the five learning sessions (see **Figure 4A**). Each group trajectory shows a main direction from left to right (along PC1) that represents the group's overall learning and off-target speed (speed in swim paths not goal-directed). For instance, the untreated Ts65Dn group trajectory reaches a maximum value of PC1 at the end of the learning phase (last learning session corresponding to their best performance level) that corresponds to initial PC1 values (learning sessions 1 and 2) of the untreated WT trajectory, indicating poor learning associated with the trisomy. Interestingly, the Ts65Dn group treated with EE-EGCG shows a trajectory that advances well into the right quadrants, attaining maximum values of PC1 that equal those reached by untreated WT at the end of the learning phase (efficient learning trajectory). There are also interesting differences in the second dimension (PC2). The most striking is that untreated WT follow an opposite trajectory to the EGCG-treated WT. Both groups reach the lowest and highest values of PC2, respectively, indicating opposite changes in swimming speed during learning upon treatment. Generally, trajectories of EGCG-treated groups have

higher values of PC2 than their untreated counterparts (with significant differences in PC2 between the EGCG treated WT and the untreated WT group, as well as between the EGCG treated Ts65Dn and the untreated Ts65Dn group on session 5, by permutation test). This indicates a general reduction in swimming speed due to EGCG treatment (data not shown).

To assess the statistical significance of these differences, we determined the amount of individual variation within each group by mapping the position of each individual on each acquisition day to the PCA plot (see Materials and Methods). As shown in Supplementary Figure 4, there is a substantial amount of individual variation across the learning sessions in all the experimental groups. In fact, the within-group variance attains 60% of the total variance (the between-group variance amounts to about 40% of the total, see Materials and Methods). Part of the between-group variance stems from the variance among learning sessions, so that the amount of between-group variance can further be decomposed into between-learning sessions (17%) and within learning-session (23%). While the former quantifies how an average group performance varies across learning sessions, the latter quantifies the average separation of the experimental groups. This separation is highly significant ($p < 10^{-4}$, permutation test, see Materials and Methods).

Figure 5 density plots show the individual variation within the Ts65Dn experimental groups during the learning process. While individuals started off from similar positions on learning session 1 (**Figure 5A**), on session 5 (**Figure 5B**) the trisomic groups spread out indicating increasing variation along the learning process. This would represent the phenotypic variability that is specifically due to learning, whereas the variation in baseline (contributed by other motivational or motor factors) is much smaller. Statistical significance of differences in learning was evaluated via a permutation test involving a t-statistic based on PC1. This analysis showed significant differences between the EE-EGCG treated and untreated Ts65Dn (p-value < 0.01) and EE-EGCG treated and EGCG treated Ts65Dn (p-value < 0.05) at the end of the learning period (session 5, **Figure 5D**), which were not observable during the first learning session (**Figure 5C**). WT mice manifested a more homogenous behavior with all groups starting from similar values in session 1 (Figure S5A) and reaching similar learning performance in session 5 (Figure S5B). WT mice showed no significant difference among treatments neither in the first session (Figure S5C) nor at the end of the learning process (Figure S5D). Significant pairwise group comparisons during learning sessions 1 and 5 based on PC1 can be found in Supplementary Tables 1, 2, respectively.

We used the same approach to analyze the data from the reversal sessions. In this case, PC1 can be interpreted as a learning composite variable explaining cognitive flexibility (Figure S6). We observed that both Ts65Dn (Figures S7A, S7B) and WT (data not shown) mice achieved higher values on PC1 along the sessions. However, there were no significant effects of the treatments within the same genotype on the last reversal session (Figure S7D), although there is a trend toward higher values of PC1 for the EE-EGCG Ts65Dn group which almost reaches significance (p-value $= 0.08$, Figure S7D). No significance differences were detected on the first reversal session (Figure S7C). It is likely that a greater number of sessions would increase the difference between double treated trisomics and untreated ones in a similar way as in the acquisition. Significant pairwise group comparisons during reversal sessions 1 and 3 based on PC1 can be found in Supplementary Tables 3, 4, respectively.

DISCUSSION

Individuals with DS undergo a progressive age-associated neurodegenerative process that resembles that of AD. Early signs of dementia in people with DS are the dysfunction of the frontal lobe and hippocampus, where amyloid first accumulates during the early stages. Cognitive symptoms of dementia in people with DS are similar to those of AD patients and include forgetfulness, impaired short-term memory, confusion, learning problems, and deficits in visuospatial organization (Lott and Dierssen, 2010). Some of these symptoms are recapitulated in DS mouse models, such as the Ts65Dn mice (Holtzman et al., 1996; Granholm et al., 2000).

The present study was aimed to investigate the potential of a combined treatment with EE and a green tea extract containing EGCG to ameliorate the hippocampal-dependent spatial learning and memory deficits in Ts65Dn mice at the age of the onset of cognitive decline. Besides the classical single-variate analysis, we applied here a novel multidimensional approach for the analysis of the effects of the different genotypes and treatments. To achieve the best discrimination between groups we used a supervised PCA involving the group medians on each acquisition session of a number of behavioral variables that are differentially modified during the learning process (see Materials and Methods). PCA has been applied to MWM analysis before. In a study from Keeley and McDonald (2015) a number of MWM navigation-related variables are mixed with variables characterizing the individuals to then identify the main contributors to overall variance as obtained by PCA. A very comprehensive MWM-related PCA study (Wolfer and Lipp, 2000) analyzed over 3000 mice from a large number of individual experiments. The approach allowed identifying a large degree of variance unrelated to spatial learning and was used to warn about oversimplified approaches disregarding variation caused by memory-unrelated effects. Our approach is rather different since it works on the group level and analyzes separately the different types of sessions. The amount of variance unrelated to genotype or treatment is taken into account by evaluating the within-group variance separately (which also enables a permutation-based significance analysis). This approach is known as discriminant analysis, and in its linear variant (LDA) has been used to classify swim paths in the MWM (Graziano et al., 2003). Our discriminant analysis based on PCA allowed depicting the 5-day trajectories of each experimental group through a space spanned by a speed-related variable and a composite learning variable. This composite learning variable reflects global treatment-induced learning differences. Traditionally, PCA does not address exact hypothesis testing, and is only used to identify which variables account for large proportions of variance in data sets, which can then inform the

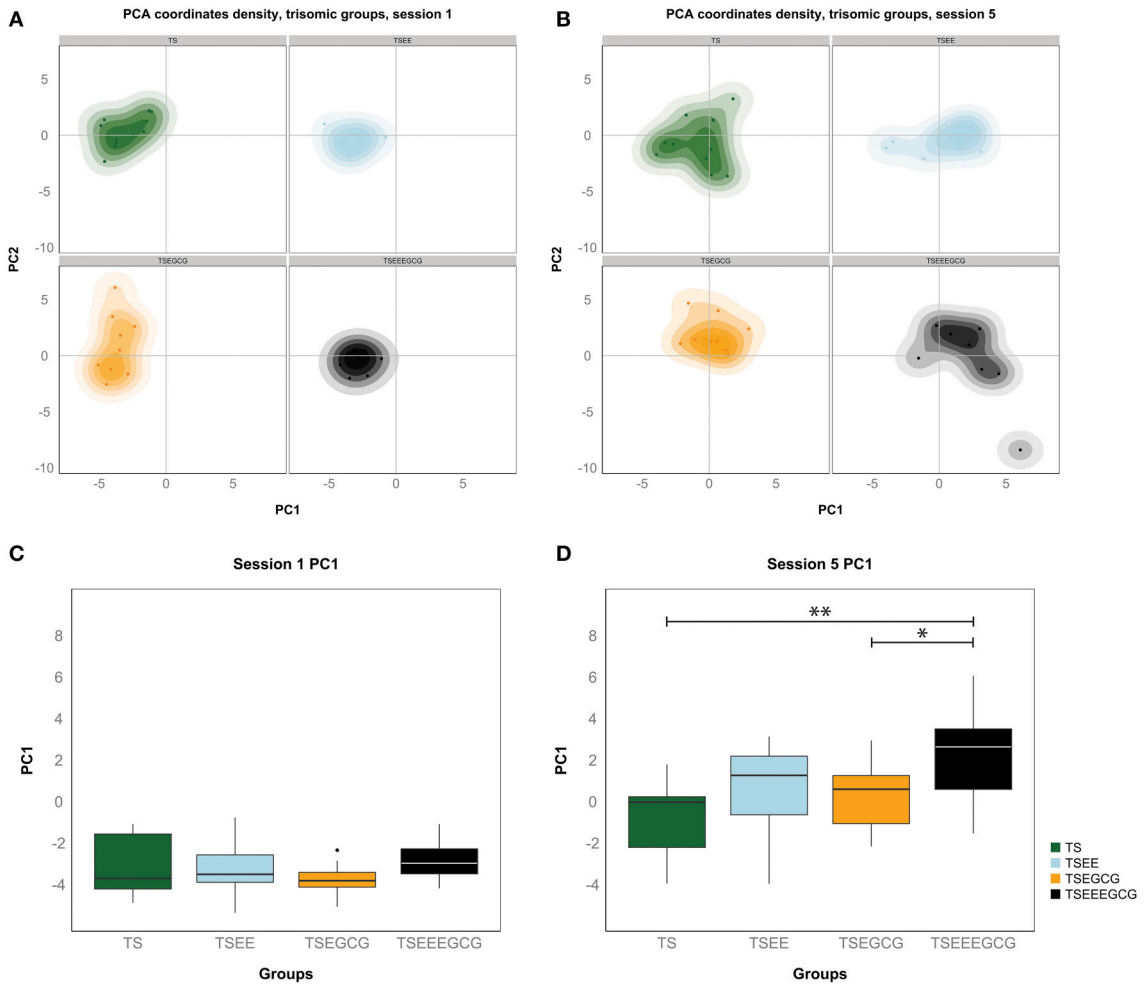

FIGURE 5 | The first principal component of the PCA (PC1) discriminates good learners from poor learners in the Ts65Dn groups. (A) Density distribution of all Ts65Dn groups for the first and the second principal components of the PCA (PC1 and PC2). On the first acquisition session all Ts65Dn groups showed a similar value on PC1, which can be interpreted as a composite variable explaining learning, indicating a comparable basal performance of all Ts65Dn animals. **(B)** TS-EE and TS-EE-EGCG mice manifested higher values of PC1 on the fifth acquisition session, at the end of the learning phase, explained by the benefits of the treatment on the learning process on these groups. On this session groups were also more spread because within group individual phenotypic differences also increased during the learning process. Boxplots of the distribution of the first principal component for each Ts65Dn group on the first **(C)** and the fifth **(D)** session of the acquisition phase. In each boxplot, the horizontal line corresponds to group median, the box edges gives the 25th and 75th percentiles and the whiskers depict minimum and maximum values to a maximum of 1.5 times the interquartile distance from the box. More extreme values are individually plotted. TS-EE-EGCG reached significant higher values on the composite learning variable than TS and TS-EGCG mice on the fifth acquisition session. Permutation test $^{*}p < 0.05$, $^{**}p < 0.01$.

choice of statistical tests among variables for ANOVA testing. Here, we applied a permutation test procedure that allowed for precise statistical significance estimations both in terms of explained variance and with respect to distances in PC1. This test has the advantage that it does not require a *post-hoc* correction for multiple comparisons or many variables. PC1 combines similarly sized contributions from six main learning-related variables that accounted for 74% of the between-group variance along the five learning sessions, and all variables, except speed, load similarly on this composite learning performance measure. This argues that all these variables capture the same amount of information concerning learning, and although in some aspects they may be redundant, they are essentially measuring slightly different learning aspects. In contrast, the second principal axis (PC2, 12% of variance) is dominated by the contribution of swimming speed and thus is mainly dependent on motor ability. However, speed also contributes to PC1, and is thus decomposed into a learning-dependent component (mice go faster to a target they have learned) and a learning-independent component (related with intrinsic motor capability). We also applied our multidimensional analysis to the reversal sessions. In this case, PC1 can also be interpreted as a composite variable explaining learning (re-learning of a new platform location related to cognitive flexibility).

As previously described, in our study both single-variate and PCA analysis showed spatial learning impairment in 6–7 months old Ts65Dn mice, as reflected along the acquisition sessions by an increased latency to reach the platform accompanied

by increased Gallagher index and thigmotactic behavior as compared to WT mice. Ts65Dn performance reached a maximum value of PC1 (composite learning measure) in the last learning session, which corresponded to initial PC1 values (learning sessions 1 and 2) of untreated WT, indicating a global learning impairment in trisomic mice. In addition, Ts65Dn mice showed an impoverished reference memory, as indicated by the significantly increased Gallagher index in the probe trial (removal session). Finally, cognitive flexibility impairment was detected in the reversal sessions as revealed by increased permanence in the previously trained quadrant, which prevented an adequate search shift to the new location of the platform. These results confirm previous studies showing that performance of Ts65Dn mice in the MWM is indicative of poor learning strategies and hippocampal-dependent learning and memory dysfunction (reviewed by Dierssen, 2012). Such impairment is detected from early stages and undergoes an age-related decline due to degenerative processes in the septo-hippocampal system (Holtzman et al., 1996; Granholm et al., 2000). Interestingly, the density plots especially of the trisomic groups on PC1 and PC2 revealed an increased within-group variance after learning (**Figure 5**), suggesting that some trisomic individuals learned better than others.

Consistently with previous findings (Martínez-Cué et al., 2002; Dierssen et al., 2003; Baamonde et al., 2011; Chakrabarti et al., 2011) we found that 1 month exposure to an enriched environment (EE) had a moderate effect on spatial learning impairment of 5–6 months old female trisomic mice, improving the efficiency in learning strategies across the acquisition sessions as shown by a reduction in the Gallagher index, in thigmotaxis and in the latency to reach the platform. These results are consistent with previous findings showing that EE induces positive though limited behavioral effects in young Ts65Dn mice. In fact, in our experiments EE had no effects on the latency to reach the platform or on the Gallagher index in WT mice, although it promoted a significant reduction in their percentage of time in the periphery along acquisition days (Figure S1), suggesting a more exploratory behavior. This is consistent with previous work in this strain (Martínez-Cué et al., 2002) that also reported reduced distances traveled in the periphery in young WT females. Different factors could account for these moderate effects of EE including gender, genetic background, or age of initiation of EE exposure.

In our study, EGCG administered for 1 month at the age of 5–6 months, did not improve spatial learning of neither WT nor Ts65Dn mice, despite the promising previous results in young Ts65Dn (De la Torre et al., 2014). Furthermore, EGCG-treated Ts65Dn group showed a trajectory in the PCA which had higher values of PC2 than untreated Ts65Dn indicating a general reduction in swimming speed during learning upon treatment. Since at 5–6 months of age Ts65Dn mice already show some age-associated cognitive decline and AD-like neuropathology (Granholm et al., 2000), it could be speculated that 1 month of treatment with EGCG at the dosage used in this study is not sufficient to reverse these effects, even though we cannot discard that a chronic treatment, initiating the administration of EGCG at earlier ages, or increasing the dosage, could restore the cognitive deficits in older trisomic animals.

Interestingly, the administration of EGCG in combination with EE was the most efficient in improving the spatial learning and memory impairment in Ts65Dn mice. EE-EGCG treatment markedly reduced escape latency, Gallagher index, and thigmotaxis. The PCA showed that EE-EGCG treated Ts65Dn group had higher values of PC1 than the rest of Ts65Dn mice groups, attaining maximum values of PC1 that were equal to those reached by untreated WT at the end of the learning phase (efficient-learning trajectory), thus suggesting a recovery of the phenotype. Regarding the reversal learning, mainly dependent on the prefrontal cortex functional integrity (De Bruin et al., 1994), according to the single-variate analysis none of the treatments were able to counteract Ts65Dn deficits. The PCA showed that EE-EGCG treatment in Ts65Dn mice was able to induce a marginal effect (p-value 0.08) at the 3rd session. The differences in EE-EGCG effects during the acquisition and the reversal sessions may be due to dysfunctions in different neural systems affecting Ts65Dn mice, involving both the hippocampus and the prefrontal cortex, which may not be equally ameliorated by the treatments.

The fact that both EE and EE-EGCG promoted similar effects, suggests that the combined administration of EE-EGCG enhanced the beneficial effect of EE. In fact, many of the effects reported for EE are overlapping those reported upon EGCG treatment, such as neuroplasticity enhancement, antioxidant activity, anti-inflammatory function, neuroprotection, promotion of the non-amyloidogenic proteolytic pathway of APP and modulation of the kinase activity of DYRK1A (for a review see Xicota et al., 2015). Specifically, EE induces a reduction of Aβ plaques (Jankowsky et al., 2005; Lazarov et al., 2005; Berardi et al., 2007; Li et al., 2013; Polito et al., 2014), of oxidative stress (Mármol et al., 2015) and increase in neurotrophins such as NGF and BDNF at the basal forebrain and other brain regions affected both in AD and DS (Ickes et al., 2000; Birch et al., 2013). Additionally, in young Ts65Dn mice short- and long-term exposure to EE has shown to reduce inhibitory neurotransmission (Begenisic et al., 2011) and rescue hippocampal cell proliferation and neurogenesis within the dentate gyrus (Chakrabarti et al., 2011). A recent paper by Gundimeda et al. (2014) shed light on other possible mechanisms as they showed that EGCG was able to potentiate the neuritogenic ability of BDNF in PC12 cells which ectopically expressed TrkB, the BDNF high affinity receptor, through the interaction with its high-affinity target 67-kDa laminin receptor (67LR), a non-integrin type cell-surface associated protein that is present in various regions of the brain. Thus, we could speculate that EGCG may enhance the beneficial effect of EE due to synergistic cellular and molecular effects between EE and EGCG since they share common functions.

High within-group variance as illustrated in density plots showed that some individuals learned better than others. In Ts65Dn mice all the treatments increased variability, indicating that some individuals are more sensitive than others to the effects of EE and the combined EE-EGCG treatment. On the other hand, a quantitative evaluation of individual variance revealed

statistically significant differences of the composite learning variable between the EE-EGCG treated Ts65Dn compared with their untreated or EGCG-treated counterparts at the end of the learning process. This indicates that EE-EGCG treatment is able to globally modify the learning related behavior.

In conclusion, we demonstrated here that combined treatment of EGCG and EE had beneficial effects on age-related cognitive impairment in Ts65Dn mice. We speculate that this may be due to synergistic cellular and molecular effects between EE and EGCG since they share common functions such as neuroplasticity enhancement, antioxidant activity, anti-inflammatory function, neuroprotection, promotion of the non-amyloidogenic proteolytic pathway of APP, and Dyrk1A kinase activity inhibition. PCA highlighted the way in which variables contributed to the variance in our data sets. As discussed above, it identified a composite learning variable and demonstrated an increased variance along the learning process within all groups and identified some trisomic individuals as more prone to the effects of EE and the combined EE-EGCG treatment than others. Overall results suggest that the combination of EGCG and EE could be an efficient therapeutic strategy in older DS individuals although there may be a large heterogeneity in the clinical outcome (responders and non-responders).

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frontiers in Behavioral Neuroscience

REVIEW
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DYRK1A, a Dosage-Sensitive Gene Involved in Neurodevelopmental Disorders, Is a Target for Drug Development in Down Syndrome

Arnaud Duchon[1,2,3,4] *and Yann Herault*[1,2,3,4,5]*

[1] Department of Translational Medicine and Neurogenetics, Institut de Génétique et de Biologie Moléculaire et Cellulaire, Illkirch, France, [2] UMR7104, Centre National de la Recherche Scientifique, Illkirch, France, [3] U964, Institut National de la Santé et de la Recherche Médicale, Illkirch, France, [4] Université de Strasbourg, Illkirch, France, [5] PHENOMIN, Institut Clinique de la Souris, Groupement d'Intérêt Économique-Centre Européen de Recherche en Biologie et en Médecine, CNRS, INSERM, Illkirch-Graffenstaden, France

Down syndrome (DS) is one of the leading causes of intellectual disability, and patients with DS face various health issues, including learning and memory deficits, congenital heart disease, Alzheimer's disease (AD), leukemia, and cancer, leading to huge medical and social costs. Remarkable advances on DS research have been made in improving cognitive function in mouse models for future therapeutic approaches in patients. Among the different approaches, DYRK1A inhibitors have emerged as promising therapeutics to reduce DS cognitive deficits. DYRK1A is a dual-specificity kinase that is overexpressed in DS and plays a key role in neurogenesis, outgrowth of axons and dendrites, neuronal trafficking and aging. Its pivotal role in the DS phenotype makes it a prime target for the development of therapeutics. Recently, disruption of *DYRK1A* has been found in Autosomal Dominant Mental Retardation 7 (MRD7), resulting in severe mental deficiency. Recent advances in the development of kinase inhibitors are expected, in the near future, to remove DS from the list of incurable diseases, providing certain conditions such as drug dosage and correct timing for the optimum long-term treatment. In addition the exact molecular and cellular mechanisms that are targeted by the inhibition of DYRK1A are still to be discovered.

Keywords: trisomy 21, neurodevelopmental disorder, mouse model, cognition, learning and memory, clinical trial, DYRK1A and kinase inhibitors

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***Correspondence:**
Yann Herault
herault@igbmc.fr

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INTRODUCTION

Since Down (1866) described patients with mental retardation and characteristic faces, Down Syndrome (DS) has been recognized as one of the most common genetic disorders leading to intellectual disability. DS results from the presence of an extra copy of all or part of chromosome 21 (Lejeune et al., 1959). The clinical presentation of DS is complex and variable. A few features occur to some degree in every individual with trisomy 21, including 100% of patients with intellectual disability, hypotonia, and cranio-facial dysmorphology, 75% with brachycephaly or 60% with epicanthic fold, 40% with congenital heart disease, and an increased incidence of leukemia in DS that is 10- to 20-fold higher than that in the general population (Antonarakis et al., 2004). The most

disabling phenotype for patients is the impaired intellectual and adaptive functioning, strongly contributed by defects in hippocampal- and prefrontal cortex-dependent functions (Pennington et al., 2003; Dierssen et al., 2009; Contestabile et al., 2010; Lott and Dierssen, 2010). These deficits are associated with both learning and short-term and long-term memory, resulting in a delay in cognitive development (Nelson et al., 2005) along with various aspects of language acquisition and comprehension (Chapman and Hesketh, 2000; Abbeduto et al., 2007). In the last decades, with better care and medical follow-up, the life expectancy of people with DS is increasing and is quickly approaching 60 years (Puri et al., 1995; Covelli et al., 2016). However, because DS patients show accelerated aging, including early onset dementia similar to Alzheimer's disease (AD) (Zigman, 2013), and the increase in life expectancy does not follow the one observed in the general population or other groups with intellectual disability (Coppus, 2013).

Down syndrome features have largely been attributed to the overexpression of specific trisomic genes (Antonarakis et al., 2004), even if non-coding element (Elton et al., 2010) or DNA methylation (Lu and Sheen, 2013) could have significant effect on DS phenotypes. Transcriptome analyses showed that between 29 and 62% of trisomic genes are overexpressed by a factor of 1.5 with some variability and depending on the cell type (Saran et al., 2003; Aït Yahya-Graison et al., 2007; Prandini et al., 2007; Sultan et al., 2007; Laffaire et al., 2009; Moldrich et al., 2009) and that trisomy has an impact on large number of genes located in domains all over the genome (Letourneau et al., 2014). A few candidate genes have been selected for therapeutic approaches because of their brain related functions, their specific pattern of expression/localization and/or their contribution to signaling pathways involved in cognitive functions. In parallel, investigation of the relationship between phenotype and genotype using a panel of rare DS patients with only partial duplication of the chromosome 21 segment led to the hypothesis of the DS chromosomal region (DCR), in which a small set of genes (between D21S55-MX1) plays a major role in the determination of DS phenotypes including the development of cognitive disabilities (McCormick et al., 1989; Rahmani et al., 1989; Korenberg, 1990; Delabar et al., 1993; Sinet et al., 1994; Korbel et al., 2009; Lyle et al., 2009). Among the 33 genes in the DCR, dual-specificity tyrosine phosphorylation-regulated kinase 1A (*DYRK1A*), which has been always found overexpressed in DS patients and mouse models (Guimera et al., 1999; Dowjat et al., 2007), has received considerable attention because of its involvement in brain functions and processes that are altered in DS, and in the early onset of neurofibrillary degeneration, β-amyloidosis, neuronal loss and AD-like phenotypes in DS (Liu et al., 2008; Wegiel et al., 2011).

DYRK1A IS A KINASE INVOLVED IN NEURODEVELOPMENTAL PROCESS AND BRAIN FUNCTION

DYRK1A is the homologue of the *Drosophila* minibrain (*mnb*) which was named from the description of the brain phenotype observed in hypomorphic mutant flies (Tejedor et al., 1995). The DYRK family includes four additional mammalian subtypes including DYRK1B, DYRK2, DYRK3, and DYRK4. DYRK proteins show little sequence homology with other kinases outside of their catalytic domains but are highly conserved across species (Becker et al., 1998). DYRK1A can catalyze its own activation through auto-phosphorylation of a single tyrosine residue in its activation loop (Lochhead et al., 2005; Soundararajan et al., 2013; Soppa and Becker, 2015).

DYRK1A is expressed during embryonic neurogenesis. First, DYRK1A is transiently detected in preneurogenic region during early mouse embryogenesis from 8 to 10.5 days postcoitum (dpc). Then *Dyrk1a* expression was observed in cycling neuronal progenitor cells of the ventricular and subventricular zones at 14.5 dpc (Hammerle et al., 2008). The authors proposed that DYRK1A controls the mouse neuronal precursor exit from differentiation, leaving the cells in a quiescent state ready to differentiate while its expression is reduced (Hammerle et al., 2011). Finally, DYRK1A is expressed and translocated from the cytoplasm to the nucleus while the dendritic tree differentiated independently in several neuronal populations (Hammerle et al., 2003, 2008). In the adult mouse, the expression of *Dyrk1a* is found in several brain regions both in the cytoplasm and the nucleus (Marti et al., 2003).

The majority of the DYRK1A protein (almost 80%) is found associated with the cytoskeletal fraction in human and mouse brain, and the remaining protein is located in the cytosolic and nuclear fractions (Marti et al., 2003; Kaczmarski et al., 2014). The phosphorylated forms of DYRK1A are specific to subcellular localization in human and mouse brain. With only one residue phosphorylated (the conserved autophosphorylation site Y321) in the cytosolic DYRK1A and multiple heterogeneous phosphorylated sites found in the cytoskeletal and nuclear DYRK1A (Kaczmarski et al., 2014). Thus the function of DYRK1A could be regulated by the action of specific kinase(s) that will influence its stability or its ability to localize to nuclear, cytosolic or cytoskeletal compartments and thus to interact with specific substrates. Indeed the nuclear accumulation of DYRK2 is controlled by the "ataxia telangiectasia mutated" (ATM) dependent phosphorylation. When phosphorylated by ATM, DYRK2 dissociates from MDM2 ("transformed mouse 3T3 cell double minute 2") and is no more degraded in the nucleus through a MDM2-dependent ubiquitination and thus could accumulate (Taira et al., 2010). This finding affecting DYRK localisation raises several questions such as whether a similar mechanism exists for DYRK1A and how it will be perturbed if there is an overdosage of the protein.

DYRK1A phosphorylates different targets depending upon its cellular localization (Marti et al., 2003; Park et al., 2009; Kaczmarski et al., 2014). It acts on a multitude of exogenous protein substrates, including transcription factors [CREB, NFAT (nuclear factor of activated T-cells), STAT3, FKHR, GLI1, RNApol2], splicing factors (cyclin L2, SF2, SF3), a translation factor (eIF2Be), miscellaneous proteins (glycogen synthase, caspase-9, Notch) or cytoskeletal target (TAU and MAP1B) and synaptic proteins (dynamin I, amphiphysin I, synaptojanin I; **Table 1**). Several targets listed here, might contribute to

TABLE 1 | Proteins that interact with MNB/DYRK1A.

Symbol	Name	Subcellular location	Biological process	Mouse Protein identification	Int.	Reference
Amph	Amphiphysin	Cytoplasm	Synaptic vesicle endocytosis	Q7TQF7	P	Murakami et al., 2006
App, b-amyloid	Amyloid precursor protein, β-amyloid precursor protein	Cytoplasm	Apoptosis, Cell adhesion, Endocytosis	P12023	P, RE	Kimura et al., 2007; Ryoo et al., 2008
Arip4	Androgen Receptor Interacting Protein 4	Nucleus	ATP, DNA and Nucleotide-binding	Q99NG0	Int.	Sitz et al., 2004
Braf	Braf transforming gene	Cytoplasm, Nucleus	ATP, Metal and Nucleotide-binding	P28028	Int.	Kelly and Rahmani, 2005
Casp-9	Cystein aspartyl protease Caspase 9	Cytoplasm, Nucleus	Apoptosis	Q8C3Q9	P	Laguna et al., 2008; Seifert et al., 2008
Ccnd1	Cyclin D1, cyclin family	Nucleus, Cytoplasm	Cell cycle and division, Transcription	P25322	P	Chen et al., 2013; Najas et al., 2015
Ccnl2	Cyclin L2, cyclin family	Nucleus	Transcription regulation	Q9JJA7	P	De Graaf et al., 2004
CreB	cAMP response element-binding protein	Nucleus	Differentiation, Transcription regulation	Q01147	P	Yang et al., 2001
Cry2	Cryptochrome Circadian Clock 2	Cytoplasm, Nucleus	Biological rhythms, Sensory transduction, Transcription regulation	Q9R194	P	Kurabayashi et al., 2010
CTD RnaP II	CTD of the RNA polymerase II	Nucleus	Transcription	P08775	P, DNA binding	Di Vona et al., 2015
Dnm1	Dynamin 1	Cytoplasm	Endocytosis	P39053	P	Chen-Hwang et al., 2002
Endophilin 1	Endophilin 1	Cytoplasm, Membrane	Endocytosis	Q62420	B	Murakami et al., 2009
Fkhr	Forkhead box O1	Cytoplasm, Nucleus	Apoptosis, Autophagy, Differentiation, Transcription regulation	Q9R1E0	P	Woods et al., 2001
Gli1	Glioma-associated oncogene 1	Cytoplasm, Nucleus	Differentiation, Transcription regulation	P47806	P	Mao et al., 2002
Grb2	Growth factor receptor bound protein 2	Cytoplasm, Nucleus	Cell differentiation	Q60631	Int.	Abekhoukh et al., 2013
Gsk3B	Glycogen synthase kinase 3 beta	Nucleus, Cytoplasm, Membrane	Differentiation, Neurogenesis	Q9WV60	P	Skurat and Dietrich, 2004
Grin2a	glutamate receptor, ionotropic, NMDA2A	Cell membrane, Cell junction	Ion transport, Transport	P35436	P	Grau et al., 2014
Hip1	Huntingtin interacting protein 1	Nucleus	Apoptosis, Differentiation, Endocytosis, Transcription regulation	Q8VD75	P	Kang et al., 2005
Kip1	Cyclin-dependent kinase inhibitor 1B	Cytoplasm	Cell cycle	P46414	P	Soppa et al., 2014
Lin52	Protein lin-52 homolog	Nucleus	Cell cycle, transcription	Q8CD94	P	Litovchick et al., 2011
Map1b	Microtubule-associated protein	Cytoplasm	Axon extension, intracellular transport	P14873	P	Scales et al., 2009
Mek1	Dual specificity mitogen-activated protein kinase kinase 1	Cytoplasm, Nucleus	ATP-binding, Nucleotide-binding	P31938	Int.	Kelly and Rahmani, 2005
Nfatc	Nuclear factor of activated T cells	Cytoplasm, Nucleus	Transcription regulation	O88942, Q60591, P97305, Q8K120	P	Arron et al., 2006; Gwack et al., 2006
Notch	Notch Signaling Pathway	Nucleus	Angiogenesis, Differentiation, Transcription	Q01705	P	Fernandez-Martinez et al., 2009
Nrsf / Rest	RE1-silencing transcription factor	Nucleus	Transcription regulation	Q8VIG1	RE	Canzonetta et al., 2008

(Continued)

TABLE 1 | Continued

P53	Transformation related protein 53	Cytoplasm, Nucleus	Apoptosis, Cell cycle, Necrosis, Transcription	P02340	P	Park et al., 2010
Phyhip	Phytanoyl-COA Hydroxylase-interacting protein	Cytoplasm	Activation of mitophagy	Q8K0S0	B	Bescond and Rahmani, 2005
Park2	Parkin	Cytoplasm, Nucleus	Autophagy, Transcription regulation	Q9WVS6	P	Im and Chung, 2015
Psen1	Presenilin1	Cytoplasm	Apoptosis, Cell adhesion, Notch signaling pathway	P49769	P	Ryu et al., 2010
Ras	GTPase Ras	Cytoplasm	Cell proliferation	Q61411, P32883, P08556	Int.	Kelly and Rahmani, 2005
Rcan1/Dscr1	Regulator of calcineurin 1	Cytoplasm, Nucleus	Calcineurin-NFAT signaling cascade	Q9JHG6	P	Song et al., 2013
Srsf1	Serine/arginine-rich splicing factor 1	Cytoplasm, Nucleus	mRNA processing, splicing and transport	Q6PDM2	P	Shi et al., 2008
Srsf2	Serine/arginine-rich splicing factor 2	Nucleus	RNA splicing	Q62093	P	Qian et al., 2011
Sept4	Septine4	Cytoplasm	GTP-binding, Nucleotide-binding	P28661	P	Sitz et al., 2008
Sf3b1/Sap155	Splicing factor 3b, subunit 1	Nucleus	mRNA processing, mRNA splicing	Q99NB9	P	De Graaf et al., 2006
Sirt1	Sirtuin 1	Cytoplasm, Nucleus	Apoptosis, Differentiation, Myogenesis, Transcription	Q923E4	P	Guo et al., 2010
Snca	a-synuclein	Cytoplasm, Nucleus	Synaptic function	O55042	P	Kim E.J. et al., 2006
Snr1	Integrase interactor 1	Nucleus	Cell cycle, Neurogenesis, Tanscription regulation	Q9Z0H3	P	Kinstrie et al., 2006
Spry2	Sprouty2	Cytoplasm	Developmental protein	Q9QXV8	P	Aranda et al., 2008
Stat3	Signal transducer and activator of transcription 3	Cytoplasm, Nucleus	Acute phase, Transcription, Transcription regulation	P42227	P	Kurabayashi et al., 2015
Synj1	Synaptojanin 1	Cytoplasm	Endocytosis	Q8CHC4	P	Chen C. et al., 2014
Tau	Microtubule-associated protein Tau	Cytoplasm	Brain development	P10637	P	Ryoo et al., 2007
Wasl	Neural Wiskott-Aldrich syndrome protein	Cytoplasm, Nucleus	Cell cycle, Cell division, Mitosis, Transcription	Q91YD9	P	Park et al., 2012
Wdr68	DDB1 and CUL4 associated factor 7	Cytoplasm, Nucleus	Ubl conjugation pathway	P61963	B	Morita et al., 2006; Miyata and Nishida, 2011

P, phosphorylation by Dyrk1A; RE, regulate expression; Int., interaction with Dyrk1A; B, binding with Dyrk1A.

the role of DYRK1A in neuronal synaptic plasticity (Wegiel et al., 2004; Aranda et al., 2008; Murakami et al., 2009, 2012). Recent studies in Cos7 cells suggest that DYRK1A is involved in the regulation of dendritic spine formation through Neural Wiskott–Aldrich syndrome protein phosphorylation (Park et al., 2012). At the synaptic level, DYRK1A could regulate synaptic vesicle endocytosis via phosphorylation of AP180, dynamin I, amphiphysin I, and synaptojanin I, as demonstrated in isolated rat brain clathrin coated vesicle (Murakami et al., 2009, 2012). Clathrin-mediated endocytosis is essential for the recycling of membrane after neurotransmitter release (Saheki and De Camilli, 2012). DYRK1A is found in the pre-synaptic compartment of the neuromuscular synapse (Arque et al., 2013). Conversely, in the Drosophila neuromuscular junction, MNB acts as a synaptic kinase that promotes efficient synaptic vesicle recycling (Chen C.K. et al., 2014). Moreover, DYRK1A phosphorylation of GRIN2A modifies the biophysical properties of GRIN1/GRIN2A, two subunits of *N*-methyl-D-aspartate receptors (NMDAR) and controls NMDAR activity in neurons, which are involved in neural development, survival, synaptic plasticity and memory processes (Grau et al., 2014).

DYRK1A Dosage and Neurodevelopmental Diseases

DYRK1A is a paradigm of a dosage sensitive gene with its underexpression, caused by heterozygous disruption or loss-of-function mutations, leading to MRD7 and its overexpression contributing to DS cognitive dysfunction. Such a variation in gene dosage could have major impact on multiprotein complex at the level of enzymatic activities or transcriptional regulation (**Figure 1**) (Veitia et al., 2008). The first MRD7 cases were translocation disrupting DYRK1A in two patients with microcephaly, severe mental retardation without speech, anxious autistic behavior, or dysmorphic features (Møller et al., 2008). Then, a second study identified patients isolated from large screen of 3,009 intellectually disabled individuals who presented a *de novo* heterozygous deletion of the last three exons of *DYRK1A* (van Bon et al., 2011). Several reports showed heterozygous mutations in *DYRK1A* in patients with multiple phenotypes, including developmental delays or intellectual disability with autism spectrum disorder, microcephaly, epileptic seizures, facial dysmorphisms and cardiac defects (Courcet et al., 2012; O'Roak et al., 2012; Bronicki et al., 2015; Ruaud et al., 2015; Van Bon et al., 2015) defining a new syndromic condition (Courcet et al., 2012; Bronicki et al., 2015; Van Bon et al., 2015). Similar neuro-developmental phenotypes (delay in eyelid and ear opening, in the appearance of the righting reflex and of the Preyer's reflex), microcephaly, locomotor activity and coordination (sensorimotor tets, balance, gait analysis, rotarod deficiency) cognition defects (delay in the startle response, spatial memory deficit in the Morris water maze and in the radial-arm water maze, memory recognition in the object recognition paradigm) and reduced dendritic tree of the layer III pyramidal cells were observed in mouse heterozygous knockout models (Fotaki et al., 2002; Benavides-Piccione et al., 2005; Dierssen and Martinez de Lagran, 2006; Arqué et al., 2008, 2009). Homozygous *Dyrk1a* knock-out (KO) mice die in utero with growth retardation, reduced body size and morphological developmental delay of the primitive organs. In an inbred genetic background, newborn heterozygous KO mice have reduced neonatal viability and decreased body size (Fotaki et al., 2002). Nevertheless, approximately 30% of the heterozygous KO mutants can survive in a mixed background. Their adult brains present increased neuronal densities in some brain regions and a specific decrease in the number of neurons in the superior colliculus, which exhibits a significant size reduction (Fotaki et al., 2002).

The role of DYRK1A in DS is deduced from several studies in mouse models. Mainly, mouse models overexpressing *Dyrk1a* or trisomic for genomic segments, homologous to Hsa21 and encompassing *Dyrk1a*, showed that *Dyrk1a* overdosage was sufficient to impair cognition with defects similar to DS people (For review see Ahn et al., 2006; Dierssen and Martinez de Lagran, 2006; Dierssen et al., 2009; Guedj et al., 2012; Herault et al., 2012). Several strategies to reduce the expression of *Dyrk1a* in DS mouse models have shown that *Dyrk1a* overdosage was necessary for DS related phenotypes. First, the normalization of *Dyrk1a* expression was achieved via adeno-associated viral delivery of a shRNA sequence specific for *Dyrk1a*. If injected in the hippocampal region, hippocampal-dependent defects in long term potentiation (LTP), a persistent increase in synaptic strength following high-frequency stimulation of a chemical synapse, and related memory performance (Morris water maze and fear conditioning) were restored in Ts65Dn mice (Altafaj et al., 2013). In addition, motor alterations were reduced after striatal injection of AAV-shRNA in transgenic mice overexpressing *Dyrk1a* (Ortiz-Abalia et al., 2008). These results demonstrated that normalization of *Dyrk1a* expression in the adult brain when neuro-developmental alterations have already occurred is sufficient to ameliorate synaptic plasticity changes via its effect on the Erk/CREB signaling pathway, that participates in the LTP induction (Thomas and Huganir, 2004), considered to be one of the major cellular mechanism sustaining learning and memory (Bliss and Collingridge, 1993; Cooke and Bliss, 2006). Second, the normalization of *Dyrk1a* copy number in trisomic mice was shown to improve hippocampal-dependent learning, presumably due to the recovery of synaptic plasticity, the enhancement of adult hippocampal cell proliferation and differentiation and/or the improvement of the balance between inhibitory and excitatory synaptic markers (Garcia-Cerro et al., 2014; Jiang et al., 2015). In addition, three copies of *Dyrk1a* was also shown to cause retinal structural and functional alterations in trisomic mice, and normalization of *Dyrk1a* copy number completely rescued both the morphological and functional visual phenotypes (Laguna et al., 2013).

Overall the phenotypes observed in MRD7 and DS affect similar areas and functions. Brain function is altered in both conditions but with different impact on intellectual abilities. As mentioned previously DS defects includes both short- and long-term memory with delay in learning and in language acquisition and comprehension (Chapman and Hesketh, 2000; Pennington et al., 2003; Nelson et al., 2005; Abbeduto et al., 2007; Dierssen et al., 2009; Contestabile et al., 2010; Lott and Dierssen, 2010)

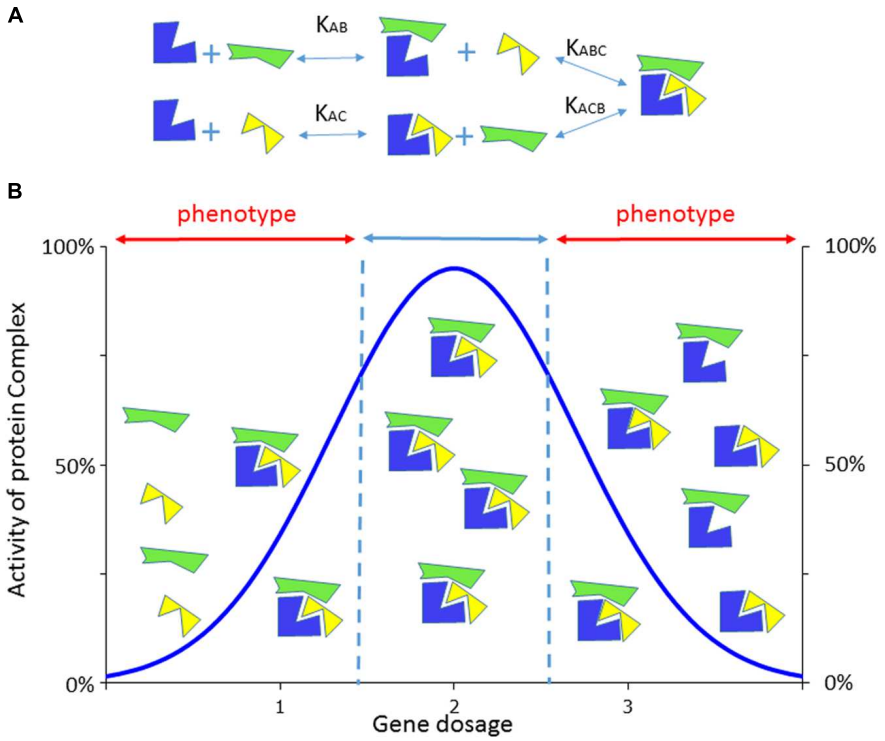

FIGURE 1 | Consequence of the dosage effect on the activity of a multiprotein complex. (A) Example of a dosage sensitive gene whose encoded protein (in blue) is able to form a tripartite complex with two partners (in yellow or green), using different constant of association/dissociation. **(B)** The formation of the complex will be altered by the level of expression of the blue protein compared to the yellow or green ones which are not dosage dependent here. With two copies of the dosage sensitive genes, complexes will be formed with the expected 100% level of activity while in only one (or three copies) are expressed the corresponding complexes will be disrupted delivering only partial activity that would lead when below (or above) a certain threshold to phenotypes. Adapted from (Veitia et al., 2008).

whereas in MRD7 autistic traits, repetitive behavior, feeding difficulties are found associated with more severe intellectual disabilities, speech delay or absence (Courcet et al., 2012; O'Roak et al., 2012; Bronicki et al., 2015; Ruaud et al., 2015). The vision is affected in both conditions but a more precise phenotypic characterization is needed in MRD7. Microcephaly is observed in MRD7 and in *Dyrk1a* heterozygous mice. Macrocephaly with more precise regional morphological changes are detected in transgenic mice overexpressing *Dyrk1a* alone (Guedj et al., 2012) but the macrocephaly is not observed in DS mouse models such as the Ts65Dn, suggesting that other genes are contributing to the DS phenotypes (Jiang et al., 2015). Accordingly DS people have a more reduced brain size, with a particular impact on the cerebellum (Pinter et al., 2001; Guihard-Costa et al., 2006), than the normal population. The microcephaly observed in MRD7 should be a direct consequence of abnormal neuronal progenitor proliferation and differentiation due to the loss-of-function of DYRK1A, and induce delay in brain development. On the opposite developmental changes do not seem to be causing the defects rescued by normalizing DYRK1A in DS models. Indeed treatment targeting DYRK1A in adult preclinical model demonstrate that the increase in DYRK1A gene dosage seems perturbed more the physiological function of DYRK1A even though the situation should be more complex in both diseases.

DYRK1A Dosage Variation and Impact on Cellular and Molecular Mechanisms

Cognitive dysfunctions observed in MRD7 and in DS result from DYRK1A misdosage during in utero development or in the adult brain. As in MRD7, the DS brain volume is smaller (Guihard-Costa et al., 2006), and this difference persists at post-natal stages (Pinter et al., 2001). Development of the superior temporal neocortex is abnormal with defect in axonal and dendritic arborization (Becker et al., 1986; Golden and Hyman, 1994). Several studies conducted in humans and DS mouse models have suggested the presence of a defect in neurogenesis and increased cell death in the hippocampus. This defect is probably due to alterations of the cell cycle in neuronal progenitors (Contestabile et al., 2007; Esposito et al., 2008; Guidi et al., 2008).

A number of studies have been conducted and demonstrate that DYRK1A dosage is key role for neurogenesis and neuronal maturation (for review see Park et al., 2009; Tejedor and Haemmerle, 2011). DYRK1A regulates proliferation and neuronal differentiation through the phosphorylation of p27Kip1 and Cyclin D1 (CCND1) (Hammerle et al., 2011; Soppa et al., 2014). *Dyrk1a* gain of function experiments were shown to stop cell proliferation with an increased number of cells expressing p27Kip1, a cyclin dependent kinase inhibitor acting as a main negative regulator of the cell cycle of neurons. Conversely its

loss-of-function triggered proliferation and cell death through a p27Kip1 downregulation (Hammerle et al., 2011). *Dyrk1a* transient expression control the neuronal precursor exit from differentiation through CCND1 and P27Kip1, leaving the cells in a quiescent state ready to differentiate while its expression reduced (Hammerle et al., 2011). DYRK1A can also regulate the G1-phase of the cell cycle of fibroblast cells through the direct phosphorylation of CCND1 (at T286), its nuclear export, degradation, and relative level with p21, all together determining the cycle entry versus exit decision. As such the G1 phase is extended in trisomic fibroblasts due to a lower CCND1 level that could be counteracted by inhibiting DYRK1A (Chen et al., 2013). The same mechanism has been found while DYRK1A controls the cell exit of ventricular neuronal progenitor cells in mouse embryos. In this compartment, *Dyrk1a* overexpression, through in utero electroporation of mouse embryos, inhibits cell cycle progression and induces premature neuronal differentiation without affecting the capacity to migrate or to differentiate in the post-natal cortex (Yabut et al., 2010). Accordingly in transgenic *Dyrk1a* mouse embryos, the G1 phase is increased in progenitor cortical stem cells due to CCND1 phosphorylation by DYRK1A, producing a deficit in cortical projection neurons that persists in postnatal stages (Najas et al., 2015). This phenotype is also present in the Ts65Dn DS mouse model which are trisomic for a large region homologous to the human chromosome 21.

Nevertheless additional mechanisms are also perturbed by change in DYRK1A dosage. DYRK1A interferes with the NFATc pathway that is critical for the regulation of vertebrate development, organogenesis and neuronal development (Graef et al., 2001; Nguyen and Di Giovanni, 2008). Overexpression of *Dyrk1a* acts synergistically with GSK3, as a priming kinase, to inhibit NFAT-dependent transcription in cortical neurons stimulated by FGF8 and heart valve elongation during development of the Ts1Cje DS models, through increase in NFAT nuclear export (Arron et al., 2006). Conversely DYRK1A and DYRK2 were found to phosphorylate NFAT regulatory domain in Drosophila (Gwack et al., 2006). As expected overexpression of both *Dyrk1a* and *Rcan1* results in delayed differentiation of neuronal progenitor cells, with a marked cell-cycle re-entry and alteration of laminar positioning. This phenomenon leads to a reduction of nuclear NFAT localization and can be rescued by expressing a constitutively active form of NFAT (Kurabayashi and Sanada, 2013). As expected interfering with *Rcan1* or *Dyrk1a* overdosage in the Ts1Cje models, or expressing the same constitutive active form of NFAT, rescue the differentiation process, found affected in the trisomic mice. As such the deregulation of the DYRK1A/RCAN1/NFAT pathway leads to developmental alterations which should impact brain size and neuronal density, two traits altered in DS.

In addition, the aberrant enhancement of astrocytic differentiation of cortical progenitor cells has been described in the Ts1Cje mouse model. It is promoted by DYRK1A acting on STAT3, a transcription factor critical for astrogliogenesis (Kurabayashi et al., 2015). This defect could be related to phenotypes observed in DS astroglia. Indeed, differentiation of human induced Pluripotent Stem cells (hiPSc)-from DS patients revealed defect in the neuronal maturation, synapse formation and neurogenesis due to specific overexpression of S100B in medium derived from hIPSc-derived astrocytes (Chen C. et al., 2014). Unfortunately, no S100b-dependent and specific phenotypes have been found so far in DS trisomic models (Yu et al., 2010a,b; Duchon et al., 2011; Belichenko et al., 2015; Jiang et al., 2015) (Duchon and Herault, personal communication) but further analysis in older individuals or to address the function of the tripartite synapse (Pereira and Furlan, 2010) might unravel additional changes.

Overall the variation in DYRK1A activity may impact the proliferation of progenitors cells, the function of the REST complex, leading to the microcephaly observed in MRD7 while in DS premature differentiation will lead to a reduced pool of mature neurons. Presumably both mechanism will contribute to the decreased brain size observed in DS, and cell density alterations found in animal models.

DYRK1A controls neuronal morphogenesis by regulating cytoskeletal dynamics, and overexpression of *Dyrk1a* in mice is sufficient to recapitulate the dendritic alterations observed in DS patients (Martinez de Lagran et al., 2012). In the adult, connectivity and plasticity are also impacted at different levels. In trisomic mice, dendritic arbor size of neocortical pyramidal cells is smaller, and the peak branching in the arbor was less complex (Dierssen et al., 2003). Total synapse density and synapse-to-neuron ratios are significantly lower in trisomic context (Kurt et al., 2004). Pre-synaptic and post-synaptic elements are significantly enlarged in the hippocampus, the motor and somatosensory cortex, the entorhinal cortex, and the medial septum (Belichenko et al., 2004). Moreover, there is a significant alteration of inhibitory synapses in the fascia dentata (Belichenko et al., 2009). These defects are probably responsible of changes in LTP and long-term depression (LTD), which have been observed in DS mouse models (Siarey et al., 1997, 1999, 2005; Kleschevnikov et al., 2004; Belichenko et al., 2007; Fernandez et al., 2007). Among other possible mechanisms, reduced *N*-methyl-D-aspartate (NMDA) receptor activation may contribute with an excessive GABAergic inhibition (Kleschevnikov et al., 2004; Costa and Grybko, 2005; Belichenko et al., 2007). In addition increase in GABAergic neurons and changes in the excitatory/inhibitory balance are observed in DS models (Altafaj et al., 2008; Grau et al., 2014; Souchet et al., 2014). Both observations are important but nothing is known at present linking DYRK1A to the glutamatergic synapse or to the GABAergic neurons.

Finally, in the later stages of life, DS patients present a neuropathology similar to AD that will evolve in dementia for 80% of patients after the age of 65 (Strydom et al., 2010; McCarron et al., 2014). This degenerative modification appears in conjunction with the presence of Aβ and tau lesions in several brain regions (Wisniewski et al., 1985). Additionally, a deficit in cholinergic neurons similar to the deficit that occurs in AD has been observed in DS during aging (Contestabile and Ciani, 2008). Even if there

is a high prevalence of dementia, not every DS patient develops the accompanying clinical symptoms (Nieuwenhuis-Mark, 2009). Interestingly, overexpression of DYRK1A and some direct targets, such as APP and MAPT, contributes to the early onset of neurofibrillary degeneration, β-amyloidosis, neuronal loss and dementia in DS (Rovelet-Lecrux et al., 2006, 2010; Salehi et al., 2006; Liu et al., 2008; Wegiel et al., 2011; Rovelet-Lecrux and Campion, 2012; Park and Chung, 2013).

DYRK1A is expressed in the nucleus and can also control the expression and interact transcription factor such as the RE1-silencing transcription factor/neuron-restrictive silencer factor REST/NRSF–SWI/SNF chromatin remodeling complex (Canzonetta et al., 2008). Through these mechanisms, increase of *Dyrk1a* gene dosage induces a SWI/SNF-linked deregulation of gene clusters involved in the neuronal phenotypic traits of DS (Lepagnol-Bestel et al., 2009). *Dyrk1a* overexpression also leads to alteration in the transcriptome, and these changes affect the NMDA type glutamate receptor with an alteration of NMDA-induced calcium dynamics (Grau et al., 2014). More recently DYRK1A has been showed to act as a transcription factor, phosphorylating the carboxy terminal domain of the RNA polymerase 2 in HeLa cells (Di Vona et al., 2015). Nevertheless no evidence have been found yet for an impact of DYRK1A dosage on the direct interaction of DYRK1A with the RNApol 2 in MRD7 or DS models.

AN ERUPTION OF DYRK1A KINASE INHIBITORS

Inhibition of DYRK1A activity represents a new field of study, and a growing number of active molecules to target this protein have been isolated over the last 5–10 years (for review see Becker and Sippl, 2011; Ionescu et al., 2012; Smith et al., 2012; Tell and Hilgeroth, 2013; Becker et al., 2014; Abbassi et al., 2015).

Historically, several compounds were isolated that exhibit dual inhibition of both DYRK family members and cell division cycle-like kinase (CLK family kinases). Effectively, the CLK and DYRK kinase families are part of the CMGC group of the eukaryotic kinome; this group, named after the initials of some members, exhibits auto-phosphorylation activity in addition to phosphorylating serine and tyrosine residues of specific substrates (Aranda et al., 2011). The CLK family has been targeted for the regulation of alternative splicing. CLK family kinases are involved in controlling HIV-1 gene expression (Wong et al., 2011), hepatic gluconeogenesis (Rodgers et al., 2010), cancer (Liu et al., 2013) and neurodegenerative disease such as AD, and their inhibition can be used as a therapeutic strategy (Jain et al., 2014). Thus, many inhibitors have been developed in recent years and are still under investigation for the treatment of DS.

Several compounds with inhibitory activity were originally isolated from natural sources. For example, harmine, a β-carboline alkaloid with a pyrido[3,4-b]indole ring structure was first isolated from the South American vine *Banisteriopsis caapi*. This molecule has been shown to be an ATP-competitive inhibitor of DYRK1A *in vitro*, with less potency against other DYRK family members (Laguna et al., 2008; Gockler et al., 2009; Adayev et al., 2011). Tg(Dyrk1a) mice, which overexpress *Dyrk1a* like people with DS, treated with harmine showed a significant decrease of homocysteine and liver ERK1/2 phosphorylation (Noll et al., 2012). Additionally, harmine prevents premature maturation of neuronal progenitors isolated from Ts65Dn (Mazur-Kolecka et al., 2012). Finally, harmine or its derivatives have been shown to reduce the levels of multiple phosphorylated forms of tau protein in tau overexpressing H4 neuroglioma cells, which are important in the pathological progression of AD (Frost et al., 2011). However, β-carboline analogs possess additional properties such as the inhibition of monoamine oxidase A, a target for depression (Kim et al., 1997), but with significant drawbacks, such as their hallucinogenic properties and a plethora of psychoactive effects, which limit their use *in vivo* (Fuentes and Longo, 1971). The flavonoid epigallocatechin-3-gallate (EGCG) was the second compound used but the first to be shown to improve cognition in DS models and in humans. Flavanoids, are characterized by having a benzopyrane skeleton, with a pyrane ring bearing at least one aromatic ring. EGCG is also a natural polyphenol and is a major catechin component of green tea leaves (*Camellia sinensis*) and was identified as a non-competitive ATP inhibitor of DYRK1A (Bain et al., 2003; Adayev et al., 2006). Both EGCG and harmine can fully restore the endocytic defects of hippocampal neurons in mouse models that overexpress *Dyrk1a* (Kim et al., 2010). In human DS- iPSCs, DYRK1A inhibition by EGCG treatment during neural induction and neuronal differentiation induces an improvement in the number of neurons and promotes dendritic development (Hibaoui et al., 2014). *In vivo* and *in vitro* studies of DS mouse models treated with a green tea extract or EGCG extract demonstrated an improvement in brain structure, adult neurogenesis, synaptic plasticity and learning and memory (Xie et al., 2008; Guedj et al., 2009; Pons-Espinal et al., 2013; De la Torre et al., 2014). EGCG also regulates the expression of REST, which is a modulator of genes that encode fundamental neuronal functions, via inhibition of DYRK1A in embryonic stem cells and in the mouse cortex (Canzonetta et al., 2008). Moreover, a pilot study of EGCG in young adults with DS demonstrated effects on memory recognition, working memory and quality of life (De la Torre et al., 2014). EGCG has been demonstrated to be a safe substance after repeated administration in humans and can be easily found as a dietary supplement because of its anti-oxidant properties (Kanwar et al., 2012). A second trial (phase II) has already been conducted by Dierssen and coworkers, and the results are highly anticipated. However, some problems with this compound remain, such as its complex pharmacokinetic properties, poor bioavailability, multiple and heterogeneous effects on signaling pathways and the degree of purity of the commercially available compound (Lambert et al., 2007; Kanwar et al., 2012; Lorenz, 2013). In addition, EGCG was found to have a low inhibitory effect on cannabinoid receptor 1 (CNR1) activity (Korte et al., 2010), which could have negative consequences for long-term treatment as the well-known rimonabant, an inverse agonist directing CNR1. Rimonabant was used as an anorectic and antiobesity

drug and was removed from the market after reports of severe depression and suicide in treated people (Thomas et al., 2014). Thus, it is essential to concentrate future efforts on finding a more specific DYRK1A inhibitor, with no interference with CNR1.

Other inhibitors were isolated from natural sources. This is the case for several marine alkaloids, such as variolins, a family containing a central pyrido[3′,2′:4,5]pyrrolo[1,2-c] pyrimidine core substituted with a 2-aminopyrimidine ring and meridianins, and a family of 3-(2-amino-pyrimidine)indoles (Gompel et al., 2004; Giraud et al., 2011; Tahtouh et al., 2012) as well as meriolins, which are a chemical hybrid between the natural products meridianins and variolins (Echalier et al., 2008). Olomoucine is one of the first CDK inhibitors to be developed, and two of its derivatives, family members of 2,6,9-trisubstituted purines roscovitine and purvalanol, also act on DYRK1A (Vesely et al., 1994; Gray et al., 1999; Bain et al., 2003). Staurosporine belongs to the class of indolocarbazoles, which bear a single sugar residue bound to both indole nitrogens, and was originally isolated from the bacterium *Streptomyces stauroporus*. This compound has exhibited potential as an inhibitor of DYRK1A, but its inhibitory activity was not selective enough among a large array of kinases (Sanchez et al., 2009).

Many inhibitors of DYRK1A kinase activity that have been developed so far are type I inhibitors; i.e., they target the ATP binding site of the kinase in its active conformation when the activation loop is phosphorylated. They have a chemical structure close to the adenine nucleus like many nitrogen heterocyclic compounds such as quinoline, quinazolines, pyrimidines, pyrrolopyrimidines, pyrrolopyridines, and pyrazolopyrimidines. These heteroaromatic cores are often capable of highly efficient binding to proteins because of their shape and hydrophobic nature. Their advantages are their lack of flexibility combined with hydrogen bonding potential from their heteroatoms that can provide a level of target selectivity. Additionally, a rapid exploration of the effect of adding different substituents is facilitated by the applicability of parallelizable reactions, and finally, two or more substitution positions can be explored without the complication of introducing a stereocenter. Their major disadvantages are their hydrophobic nature and flat shape, resulting in low aqueous solubility (Pitt et al., 2009). These inhibitors have higher potency on substrate phosphorylation than on autophosphorylation. Serine and threonine phosphorylation of substrates is inhibited with lower impact on autophosphorylation and the remaining tyrosine kinase activity. Such a difference may reflect the accessibility of the inhibitor target site. DYRK1A kinase activity is closely regulated, with two successive conformational states proposed: the first immature state allows the enzyme to act on the tyrosine residue, while the second more mature state, being irreversible, reacts with the serine/threonine residue ($R(X_{1-2})S/TP$) (Lochhead et al., 2005). The two-states model was enriched with the two conformations having slight differences in reacting with tyrosine or serine/threonine, with the immature state having lower catalytic activity and different equilibrium for the amino acid than the mature state (Walte et al., 2013). Additionally, several inhibitors have been synthesized, such as Tg003 (Muraki et al., 2004) and INDY (Ogawa et al., 2010) two benzothiazole derivatives. For example, DANDY, a new 3,5-diaryl-7-azaindole that demonstrates potent inhibition against DYRK1A kinase activity (Gourdain et al., 2013). HCD160 and its derivatives (Kim N.D. et al., 2006; Koo et al., 2009), a series of substituted 6-arylquinazolin-4-amines (Mott et al., 2009; Rosenthal et al., 2011), a new 3-(6-hydroxyindol-2-yl)-5-(phenyl)pyridine (Kassis et al., 2011), a series of aryl-substituted aminopyrimidines (Coombs et al., 2013), a new 7-substituted pyrido[2′,3′:4,5]furo[3,2-d]pyrimidin-4-amines (Deau et al., 2013), an 8-arylpyrido[30′20′:4,5]thieno[3,2-d]pyrimidin-4-amines (Loidreau et al., 2015), and a series of hydroxybenzothiophene ketones (Smith et al., 2012; Schmitt et al., 2014). To date, no DYRK-specific or CLK-specific inhibitors have been reported, certainly because there is a high degree of conservation of the ATP binding site inside the CLK and DYRK kinase families. Nevertheless novel interesting compounds are in development (Rüben et al., 2015). Even if some cellular side effects have been reported for several of the listed compounds, none of them have yet been tested in DS models. Moreover, the activity

TABLE 2 | Proteins that regulate Dyrk1A activity.

Symbol	Name	Subcellular location	Biological process	Mouse Protein identification	Interaction	Reference
14-3-3	14-3-3 proteins	Nucleus	Brain development	Q9CQV8, P62259, P61982, P68510, P68254, P63101, O70456	Binding	Kim et al., 2004; Alvarez et al., 2007
Fgfb	Basic fibroblast growth factor	Nucleus	Angiogenesis, Differentiation	P15655	Not described	Yang et al., 2001
E1A	Human adenovirus early region 1A	Nucleus	Oncoprotein		Protein Interaction	Zhang et al., 2001
E2f1	Transcription factor E2F1	Nucleus	Apoptosis, Cell cycle, Transcription	Q61501	Not described	Maenz et al., 2008
Lats2	Large tumor suppressor 2	Cytoplasm, Nucleus	Cell cycle, Cell division, Mitosis	Q7TSJ6	P	Tschop et al., 2011

of these compounds is usually stronger on CLK kinases, excluding varioline B, leucettine 41 and harmine (Tahtouh et al., 2012).

Recent progress on more specific and selective inhibitor has already been made. For example, we note the development of a new bioluminescent reporter assay for evaluation of DYRK1A inhibitors. This system led to the identification of (*Z*)-5-[(2,3-dihydrobenzofuran-5-yl)methylene]-2-iminothiazolidin-4-one (referred to as CaNDY: CDC37 association inhibitor for DYRK1A) as a strong inhibitor of DYRK family kinases. In addition to inhibition potential, CaNDY decreases DYRK1A molecules in cells, thus efficiently suppressing DYRK1A activity compared to simple inhibition of kinase activity (Sonamoto et al., 2015). Substituted quinazolines are a common pharmacophore for ATP-competitive kinase inhibitors, and the 5 novel thiazolo[5,4-f]quinazoline derivatives that have been synthesized (EHT 5372, 6840, 1610, 9851 and 3356) are among the most potent DYRK1A/1B inhibitors disclosed to date. In particular, they are more potent than NCGC-00189310 (Rosenthal et al., 2011) and leucettine L41 (Tahtouh et al., 2012), the two most active reference inhibitors tested during the screening campaign (Foucourt et al., 2014; Coutadeur et al., 2015). In addition, an indirubin derivative was inactive toward cyclin-dependent kinase 5 (CDK5), GSK3β, and casein kinase 1 (CK1) while exhibiting good selectivity and affinity for DYRK kinases (Myrianthopoulos et al., 2013). Consequently, the development of type II or III inhibitors, non-ATP-mimetics that are anchored to more diverse regions of the ATP binding site, may result in more selective inhibitors while also targeting the premature DYRK1A kinase. For example KHCB19 is a dichloroindolyl enaminonitrile derived from bauerine C, a β-carboline alkaloid originally isolated from the blue-green alga *Dichothrix baueriana*. This compound exhibits a unique 'non-ATP mimetic like' binding mode to CLK1 and also acts on DYRK1A (Fedorov et al., 2011). The non-ATP competitive inhibitors, called type II and type III inhibitors act by inducing a conformational shift in the target enzyme such that the kinase is no longer able to function. This unusual binding mode highlights the opportunity to develop very potent and specific inhibitors with new chemical profiles because they offer the possibility to overcome the major problem of the type I inhibitor (Garuti et al., 2010). Nevertheless even if a more potent and specific inhibitor would be better, too much inhibition may be deleterious as in the case of loss-of-function mutation of *DYRK1A* in MRD7. In addition DYRK1A specific inhibition on substrates phosphorylation versus autophosphorylation would be interesting to determine. A specific substrate kinase inhibition may have a more beneficial effect rather than targeting the autophosphorylation. The situation is even more complex considering that the target site of the inhibitor might have different accessibility depending on the two conformations of DYRK1A.

Modulation of DYRK1A in MRD7 by increasing its activity would be a good strategy to alleviate the severe cognitive deficits present in the disease, in particular for loss-of-function mutations. Nevertheless only a few proteins are known to increase DYRK1A activity (see **Table 2**) and no DYRK1A compound activator has been described to date. Thus further work is needed to investigate such a strategy for MRD7.

CONCLUSION

Major steps have been achieved to establish the role of DYRK1A in intellectual disabilities such as DS and MRD7 syndromic condition involving heterozygous loss-of-function mutations affecting DYRK1A. These two neurodevelopmental disorders share common features resulting from the alteration of DYRK1A-controlled mechanisms such as neuronal proliferation and differentiation. However, many questions on DYRK1A inhibitors remain unanswered. Are type 1 DYRK1A inhibitors blocking the ATP binding site better suited than type 2 or 3 inhibitors selective for a particular conformation of DYRK1A? What are the cellular targets of DYRK1A: GABAergic or glutamatergic neurons, glial cells?

Undeniably DYRK1A inhibitors represent a promising class of molecules to improve cognitive deficits in people with DS. The pilot clinical trials in adults treated with EGCG is promising, showing a modest gain of cognition (De la Torre et al., 2014). Perinatal treatment during earlier phases of brain development is attractive for improving synaptic plasticity, keeping in mind that DYK1A plays an important role in brain growth by controlling neuronal proliferation and differentiation. Additional studies will be needed to evaluate the use of DYRK1A inhibitors during perinatal periods.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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