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Effects of Maternal Choline Supplementation on the Septohippocampal Cholinergic System in the Ts65Dn Mouse Model of Down Syndrome

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Abstract

Down syndrome (DS), caused by trisomy of chromosome 21, is marked by intellectual disability (ID) and early onset of Alzheimer's disease (AD) neuropathology including hippocampal cholinergic projection system degeneration. Here we determined the effects of age and maternal choline supplementation (MCS) on hippocampal cholinergic deficits in Ts65Dn mice. Ts65Dn mice and disomic (2N) littermates sacrificed at ages 6–8 and 14–18 mos were used for an aging study, and Ts65Dn and 2N mice derived from Ts65Dn dams were maintained on either a choline-supplemented or a choline-controlled diet (conception to weaning) and examined at 14–18 mos for MCS studies. In the latter, mice were behaviorally tested on the radial arm Morris water maze (RAWM) and hippocampal tissue was examined for intensity of choline acetyltransferase (ChAT) immunoreactivity. Hippocampal ChAT activity was evaluated in a separate cohort. ChAT-positive fiber innervation was significantly higher in the hippocampus and dentate gyrus in Ts65Dn mice compared with 2N mice, independent of age or maternal diet. Similarly, hippocampal ChAT activity was significantly elevated in TS65Dn mice compared to 2N mice, independent of

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CONFLICT OF INTEREST

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maternal diet. A significant increase with age was seen in hippocampal cholinergic innervation of 2N mice, but not Ts65Dn mice. Degree of ChAT intensity correlated negatively with spatial memory ability in unsupplemented 2N and Ts65Dn mice, but positively in MCS 2N mice. The increased innervation produced by MCS appears to improve hippocampal function, making this a therapy that may be exploited for future translational approaches in human DS.

Keywords

aging; cholinergic; Down syndrome; hippocampus; intellectual disability; maternal choline supplementation; spatial memory; Ts65Dn mice

1. INTRODUCTION

Down syndrome (DS) is a multi-faceted condition caused by triplication of human chromosome 21 (HSA21) that can result in defective function within cardiac, respiratory, endocrine, gastrointestinal, immunological, and central nervous systems; in addition, DS presents as cognitive problems in the domains of learning, language, and memory, forming an overall pervasive intellectual disability (ID) [1,2]. A premature aging phenotype is also observed that includes classic neuropathologic features of Alzheimer's disease (AD): amyloid plaques, neurofibrillary tangles, and degeneration of basal forebrain cholinergic neuron (BFCN) systems [3,4]. BFCNs provide the major cholinergic innervation to the entire cortical mantle and the hippocampus [5,6], and dysfunction in this system results in significant cognitive difficulties. It is estimated that individuals with DS show a clinical presentation of dementia in ~50 % of cases over the age of fifty [7–11], although this may be an underestimation, as assessing cognitive decline in persons with ID is difficult and sometimes equivocal.

In both AD and DS, there is a decrease in cell size and density of BFCNs, culminating in a reduction in cholinergic innervation within the hippocampus [12–19]. There have been several attempts to mimic a DS-like phenotype in animal and cellular models. Although initial attempts with full trisomy models were not successful, segmental trisomic models of DS have proven to be extremely useful for DS and AD research. Specifically, key aspects of DS neuropathology, is recapitulated in the Ts65Dn mouse, making it a valuable translational model of DS and AD [20–27]. Ts65Dn mice are trisomic for a segmental region of murine chromosome 16 (MMU16) orthologous to part of human chromosome 21 (HSA21) [28–30]. For example, age-related alterations in the BFCN system of Ts65Dn mice have been reported for the septohippocampal cholinergic projection system, including neuronal loss [20,26,31,32], altered patterns of cholinergic innervation within cholinceptive regions, and changes in activity levels of choline acetyltransferase (ChAT), acetylcholinesterase (AChE), and high-affinity choline uptake (HACU) [33–36].

Recently, our group reported the effect of dietary maternal choline supplementation (MCS) on hippocampal-dependent behavior and BFCNs in adult (13–17 mos) Ts65Dn mice and 2N offspring [37]. Spatial mapping was impaired in unsupplemented Ts65Dn mice relative to 2N littermates, a significantly lower number and density of cholinergic neurons within the medial septal (MS) and vertical limb of the diagonal band (VDB) complex was seen in

unsupplemented Ts65Dn mice compared with treatment-matched 2N mice, and MCS improved spatial learning and increased number and size of cholinergic MS and VDB neurons in Ts65Dn offspring [37]. The density and number of cholinergic MS neurons also correlated with spatial memory proficiency in adult Ts65Dn mice [37].

While many of the triplicated genes in human DS and the Ts65Dn mouse model are implicated in ID and age-related BFCN pathology, no specific metabolic pathway or process has been definitively shown to be responsible for the premature aging pathology observed in DS humans or trisomic murine models. This has made it difficult to develop an effective treatment strategy for the ID and early onset AD-like pathology of DS. A potential treatment approach involves maternal dietary supplementation with choline, an essential nutrient involved in neural tube closure, organogenesis, central nervous system cell membrane synthesis, and methylation-dependent gene expression [36,38–44]. MCS has both immediate and long-term beneficial effects in healthy disomic rats and mice [44–57], and in rodent models of prenatal ethanol exposure, Rett syndrome, and status epilepticus [58–63]. To explore the effects of early choline supplementation on cholinergic innervation and activity in the hippocampus of the adult brain, we used a MCS paradigm and studied adult male Ts65Dn and 2N offspring. Since cholinergic MS/VDB neurons innervate the hippocampus [5,6,64,65], it is likely that disruption of this memory circuit underlies some of the pervasive cognitive deficits seen in DS as well as age-related cognitive decline. While several independent groups have reported atrophic changes in Ts65Dn mice beginning as early as 2 mos of age, including decreased BFCN size, endosomal abnormalities, and defective retrograde transport, overall BFCN loss and decreased hippocampal cholinergic innervation is not seen consistently until after 10 mos [20–26]. In the present study, we determined the effects of MCS, provided via dietary supplementation from conception to weaning, upon septohippocampal cholinergic innervation and activity in adult Ts65Dn and 2N offspring.

2. MATERIALS AND METHODS

2.1. Animals

2.1.1. Aging Cohort—Male Ts65Dn and 2N mice were obtained from Jackson Laboratories (Bar Harbor, Maine, USA) at 1 mo and 10 mos of age. Both genotypes are derived from a cross of female Ts65Dn and male C57BL/6J $\times$ B6EiC3SnF1/J mice, and only mice that were not homozygous for *Pde6b^{rd1}*, a gene that causes retinal deterioration and blindness, were used [26,66]. Final group numbers were: thirteen 2N and ten Ts65Dn mice between 6.5–8.1 mos of age, and thirteen 2N and eleven Ts65Dn mice between 13.9–18.4 mos of age (see Table 1). Groups of young-middle aged adult mice are referred to as 6–8-month-old mice and the two late middle-aged as 14–18-month-old mice.

2.1.2. Choline Supplementation Cohorts—For the choline supplementation study, additional breeder pairs (Ts65Dn female and C57BL/6J Eicher $\times$ C3H/HeSnJ F1 male mice) were purchased from Jackson Laboratories, and mated and housed at Cornell University (Ithaca, NY, USA). For genotyping, tail snips or ear punches were taken at weaning and sent to Jackson laboratories where genotype as well as determination of *Pde6b^{rd1}* homozygosity was discovered by quantitative polymerase chain reaction (qPCR).

After weaning at postnatal day 21, male pups were group-housed; genotypes were mixed as animals were coded and researchers experimentally blind to the genotype until post-data analysis. Mice were maintained on an inverse light:dark cycle and 6–8 wk prior to termination mice were singly housed. Separate cohorts were used to determine changes in ChAT-positive hippocampal innervation (n=11, aged 13.7–17.8 mos) and ChAT activity levels (n= 15–18 mice, aged 14.7–18.6 mos; see Table 1). Behavioral data reported in this study was derived from the same cohort of male mice processed for immunohistochemistry.

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

2.2. Choline Supplementation

Dams were randomly assigned to either a choline-controlled rodent chow diet (AIN-93G with 1.1 g/kg choline chloride; Dyets Inc., Bethlehem, PA, USA) or a rodent chow diet with choline supplementation (AIN-93G with 5.0 g/kg choline chloride; 5.0 g/kg; Dyets Inc.) as previously reported [37,67–69]. The choline-supplemented diet provided approximately 4.5 times the concentration of choline in the normal diet, within the range of dietary variation observed in the human population [70]. Administration of choline as an oral supplement or through consumption of foods high in choline moiety content, increases plasma and cerebral spinal fluid levels of choline [71–73]. Moreover, increases in plasma choline levels directly correlate with increases in brain choline and acetylcholine (ACh) levels [74,75]. The breeder pairs were provided ad libitum access to their assigned diet. Offspring remained with their original dams until weaning; and postweaning, male offspring were fed the control diet, once *per diem* with an amount determined by weight (to avoid obesity).

2.3. Tissue Preparation

Mice were deeply anesthetized with ketamine (85 mg/kg)/xylazine (13 mg/kg) via intraperitoneal injection, and perfused transcardially with 0.9 % saline (50 ml), followed by 4% paraformaldehyde (50 ml) in phosphate buffer (PB; 0.1M; pH = 7.4) as previously described [67,68]. Ages at sacrifice are listed in Table 1. For stereological and ChAT luminosity measurements, brains were extracted from the calvaria, postfixed for 24 h in the same fixative, and placed in a 30% sucrose PB solution at 4 °C until sectioning. Brains were then cut at 40 µm thickness in the coronal plane on a sliding freezing microtome and stored at 4 °C in a cryoprotectant solution (30 % ethylene glycol, 30 % glycerol, in 0.1 M PB). For ChAT enzymatic assay, mice were transcardially perfused with a 0.9 % saline solution, brains extracted, hippocampus dissected out on wet ice, and stored at –80° C until analysis.

2.4. Immunohistochemistry

Immunohistochemistry was performed as described previously [76]. Briefly, a full series of sections from each animal was immunolabeled with a goat polyclonal antibody for choline acetyltransferase (ChAT; 1:1000 dilution; Millipore, Billerica, MA, USA), the non-rate-limiting enzyme for acetylcholine synthesis and a specific marker for cholinergic neurons [77]. Tissue was washed in phosphate buffer (PB, pH 7.2, 1X) to remove excess cryoprotectant, rinsed in Tris-buffered saline (TBS, pH 7.4, 1X), and incubated in sodium

metaperiodate to inhibit endogenous peroxidase activity. Tissue was then washed in TBS containing 0.25 % Triton X-100 to enhance primary antibody penetrance. This was followed by 1 h incubation in a blocking solution consisting of 3% horse serum in TBS/Triton X-100, then incubation overnight at room temperature with the ChAT primary antibody in a solution of TBS/Triton X-100 with 1 % horse serum. All washes and incubations were carried out at room temperature on a shaker table.

Following overnight incubation, sections were washed in TBS and incubated for 1 h with a biotinylated secondary antibody (anti-goat IgG, host horse, 1:200, Vector Laboratories, Inc., Burlingame, CA, USA), allowing for signal amplification by a proceeding incubation for 1 h in avidin-biotin-complex solution (Elite kit, Vector Laboratories, Inc.). Prior to chromogen reaction, tissue was washed in a sodium imidazole acetate buffer (0.68 g imidazole, 6.8 g sodium acetate trihydrate per 1 L distilled water, pH 7.4 with glacial acetic acid). Antibody immunolabeling was visualized with 0.05 % 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich, St. Louis, MO, USA) with 1 % nickel (II) ammonium sulfate hexahydrate and 0.0015 % H₂O₂. The reaction was terminated using fresh imidazole-acetate buffer solution, and sections were rinsed in PB, mounted on chrome-alum-gelatin-subbed slides, dried overnight at room temperature, dehydrated in a graded series of ethanol, cleared in xylenes, and cover-slipped with distyrene/dibutylphthalate (plasticizer)/xylene (DPX) mounting medium. Immunolabeling and mounting of sections was performed in one set, with animals randomized across baskets to reduce batch-to-batch variation, preclude group staining variation, and reduce overall experimental variance. Immunohistochemical controls with the omission of primary antibodies were conducted on tissue processed at the same time as the ChAT-immunolabeled sections. All procedures were performed blind to genotype and treatment.

2.5. ChAT Hippocampal Luminosity Measurements

The intensity of ChAT-immunolabeling was determined by tracing the hippocampus and dentate gyrus unilaterally at three points along its rostrocaudal axis using an X1 lens (n.a. 0.04). Photomicrographs were then taken with an X10 lens (n.a. 0.45) and montaged using Virtual Slice software (Stereo Investigator, MicroBrightField, Inc.) with re-focusing at every three sites [67]. For this procedure, all photomicrographs were taken at the same level of illumination, and a background image taken from a blank area of the glass slide was used to correct for alterations in luminosity across the plane of focus.

Intensity of ChAT-immunoreactive fibers was measured at 23 sites using ImageJ software (1.45s, 1.6.0–20, 32-bit) [78] including nine sites in the dentate gyrus (DG), eleven sites across the hippocampus proper, and three background sites within the corpus callosum white matter dorsal to the hippocampus. There were no differences between corpus callosum background measures for any groups or sections, all within < 1 % of each other average (0.96 %, or 2.45 units on a scale of 0–255). The three background measurements were averaged per section, and each hippocampal ChAT intensity measurement was divided by the average background measurement for that section. Because no difference was seen between the ventral and dorsal blades of the dentate gyrus, the data were pooled; for example, DG inner molecular layer (IML) intensity was calculated by averaging dorsal and

ventral inner molecular layer values, and DG outer molecular layer (OML) intensity was calculated by averaging dorsal and ventral outer molecular layer values. Averaging all measurements across the DG and hippocampus proper derived total hippocampal ChAT intensity. Hippocampus proper intensity was determined by averaging CA2/3, CA1/2, and CA1 measurements. All calculations were performed for each subject, prior to calculating group values. Data are plotted as inverse values with 1.0 representing saturation with white light (pixel value 255) and values > 1.0 representative of increased ChAT intensity (pixel values < 255).

2.6. ChAT Radioenzymatic Assay

Hippocampus from Ts65Dn mice and age-matched 2N littermates at 14–18 mos (see Table 1) were processed for determination of ChAT enzyme activity using a modification of the Fonnum method [79–81]. Although the Fromun assay is a standard in the field for ChAT activity, the procedure has limitations and may detect carnitine acetyltransferase (CrAT) activity [82]. Frozen tissue from hippocampal dissections was homogenized using high frequency sonication in a solution containing 0.5 % Triton X-100 and 10 mM ethylenediaminetetraacetic acid (EDTA). Briefly, 5 μ l of tissue homogenate was combined with C-14 labeled acetyl Co-A (New England Nuclear, Boston, MA, USA), incubation buffer (100 mM sodium phosphate, 600 mM NaCl, 20 mM choline chloride, 10 mM disodium EDTA, pH7.4), and physostigmine (20 mM, Sigma-Aldrich, St. Louis, MO, USA). After 30 min of incubation at 37° C in a water bath, the reaction was stopped by the addition of 4 ml of 10 mM phosphate buffer (pH 7.4). Subsequently, 1.6 ml of acetonitrile/tetraphenylboron mixture and 8 ml of scintillation fluid were added to cause phase separation. After the samples stabilized for 24 h, they were counted in a scintillation counter. Protein content of the samples was determined using a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). ChAT activity was expressed as μ mol/h/g protein. Samples were coded, and all assays performed in triplicate by a technician blinded to genotype and treatment groups.

2.7. Statistical Analysis

For hippocampal ChAT intensity analysis, the nonparametric Mann Whitney U test was used for determining differences between groups, and the Friedman test was used for within group comparisons; as such, all averages are presented as median. Statistics were conducted using Excel (14.0.6129.5000, Microsoft, Redmond, WA, USA) and SPSS (PASW Statistics 18, release 18.0.0, IBM, Armonk, NY, USA). For hippocampal ChAT radioenzymatic data analysis, the Kruskal-Wallis test was used. Statistical significance set at $p < 0.05$.

For behavioral analysis performed in a separate study [37], the 15 sessions were divided into 5 3-day blocks with mean number of errors committed per trial, averaged across blocks, established as the dependent measure. Nonparametric correlations were calculated using the Statistical Analysis System (version 9.3; SAS Institute, Cary, NC, USA) and are presented as Spearman's rank correlation coefficient (r_s). The alpha level was set at 0.05.

3. RESULTS

3.1. Hippocampal Cholinergic Intensity in Ts65Dn and 2N Mice During Aging

Examination of average ChAT intensity across the hippocampus and dentate gyrus was significantly increased by 65 % in Ts65Dn mice compared with 2N mice at 6–8 mos ($p < 0.001$, Figure 1). A significant 33 % age-related increase was seen in 2N mice at 14–18 mos compared with 2N mice at 6–8 mos ($p < 0.05$). No parallel change in ChAT intensity with age was observed in Ts65Dn mice, although a nonsignificant 21% increase in ChAT immunostaining was observed within Ts65Dn mice compared with 2N mice at 14–18 mos (Figure 1).

ChAT intensity within individual hippocampal laminae showed elevated levels in Ts65Dn mice at 6–8 mos compared with age-matched 2N mice. These findings were largely driven by increased ChAT immunoreactivity within the rostral and middle sections across each hippocampal laminae examined (Table 2). In contrast, an age-related increase in a total ChAT intensity was seen in 2N mice, which was driven primarily by ChAT intensity in the stratum pyramidale in the rostral CA1–CA3 regions and by the granule and molecular layers of the dentate gyrus (Table 2).

3.2. Elevated ChAT Intensity in the Hippocampus and Dentate Gyrus of Adult Ts65Dn Mice Independent of Maternal Diet

Similar to the aging cohort, when averaged across all laminae and subregions of the hippocampus and DG, ChAT intensity was consistently higher, on average by 19%, in unsupplemented Ts65Dn mice compared with unsupplemented 2N mice at 14–18 mos (see Table 1 and Figure 3). Mice exposed to MCS displayed a nonsignificant average 18 % increase in ChAT intensity in the rostral and middle aspects of the hippocampus of Ts65Dn mice over genotype-matched unsupplemented mice; while, no change was observed within the rostral, middle, or caudal hippocampus of 2N mice exposed to MCS. In MCS offspring, ChAT intensity remained elevated in Ts65Dn mice over treatment-matched 2N mice, with a significantly higher level found in the caudal hippocampus ($p < 0.05$, Figure 3).

3.3. ChAT Intensity in the Hippocampus Proper

When individual laminae of the hippocampus were examined, patterns of innervation were comparable across the different hippocampal layers between Ts65Dn and 2N mice in both maternal diet conditions (Figure 3). For all groups, levels of ChAT intensity was highest in the stratum pyramidale (s.pyr.) and lowest in the stratum radiatum (s.r.) when CA1–CA3 regions were averaged. By contrast, ChAT intensity was higher in all laminae for CA3 compared to CA1 for all groups (Figure 3). Even in the s.pyr. layer of the rostral hippocampus where this effect was lowest in 2N and Ts65Dn unsupplemented mice, there was a significant difference between CA3 and CA1 (2N $p < 0.001$, Ts65Dn $p < 0.01$, Friedman test). MCS did not alter the pattern of innervation, but nonsignificantly increased the level of ChAT intensity in all CA1–CA3 laminae for both genotypes, markedly in the rostral hippocampus (Figure 3, Table 3).

3.4. ChAT Intensity in the Dentate Gyrus

Higher levels of ChAT intensity were seen in Ts65Dn mice compared with treatment-matched 2N mice in granule, hilus, and molecular layers of the DG (significance shown in Figure 4, where 1 denotes significance between genotypes in unsupplemented mice and 4 in supplemented mice). A nonsignificant average 23 % increase in ChAT intensity was seen in the granule layer of Ts65Dn mice that received MCS, with little change observed in 2N offspring exposed to MCS (Figure 4). ChAT intensity was consistently elevated in the OML compared with the IML for all groups, with no effect of genotype or treatment observed (Figure 4).

3.5. Examination of ChAT Intensity in the Dentate Gyrus Inner and Outer Molecular Layers

Similar to the hippocampus proper (Figure 3), Ts65Dn mice had significantly higher ChAT intensity values than maternal diet-matched 2N mice for the IML (rostral and caudal unsupplemented, $p < 0.05$; middle unsupplemented, $p < 0.01$; rostral, middle, and caudal supplemented, $p < 0.01$), and in supplemented groups for the OML (rostral, middle, and caudal, $p < 0.05$; Figure 5). However, no significant change with MCS was seen for offspring of either genotype (Figure 5, Table 3).

3.6. RAWM Performance and Hippocampal ChAT Intensity

Behavioral testing of the mice used in this study were correlated with the current morphometric findings. Positive associations between mean number of maze errors and the intensity of ChAT-immunolabeling were observed in the DG OML ($r_s = 0.30$, $p = 0.049$; Figure 6), DG IML ($r_s = 0.28$, $p = 0.065$; Figure 6), and the hippocampus proper ($r_s = 0.27$, $p = 0.080$; Figure 6), indicating that greater ChAT intensity was associated with poorer spatial memory performance. However, this relationship was largely driven by unsupplemented groups where a significant positive relationship was seen between mean number of errors and ChAT intensity in the OML ($r_s = 0.83$, $p = 0.003$) and IML ($r_s = 0.64$, $p = 0.044$) of the DG, as well as the hippocampus proper ($r_s = 0.67$, $p = 0.034$) of 2N unsupplemented mice ($r_s = 0.67$, $p = 0.035$), and in the OML ($r_s = 0.67$, $p = 0.023$) and IML ($r_s = 0.63$, $p = 0.039$) of the DG, and the hippocampus proper ($r_s = 0.63$, $p = 0.038$) of unsupplemented Ts65Dn mice. In contrast, supplemented 2N mice showed a negative correlation between mean number of errors and ChAT-positive intensity (OML, $r_s = -0.72$, $p = 0.013$; IML, $r_s = -0.81$, $p = 0.003$; hippocampus, $r_s = -0.76$, $p = 0.006$; Figure 6), whereas supplemented Ts65Dn mice showed no correlation (OML, $r_s = 0.26$, $p = 0.43$; IML, $r_s = 0.37$, $p = 0.25$; hippocampus, $r_s = 0.27$, $p = 0.41$; Figure 6).

3.7. Elevated ChAT Activity in the Hippocampus of Ts65Dn mice

Quantitative biochemical analysis revealed elevated hippocampal ChAT activity in the hippocampus of Ts65Dn mice compared with 2N littermates at age 14–18 mos, independent of maternal diet (unsupplemented, $p < 0.0001$; choline supplemented, $p < 0.005$; Figure 7).

4. DISCUSSION

4.1. Aging

The present findings did not reveal elevated hippocampal ChAT intensity with age in Ts65Dn mice, while an age-related increase in total hippocampal ChAT intensity was observed in 2N littermates. This increase in a hippocampal cholinergic marker is similar to that seen in people with a clinical diagnosis of mild cognitive impairment (MCI), an assumed prodromal stage of AD, prior to frank BFCN loss [83–87].

Ts65Dn mice are born with intact BFCNs in the MS, which provide cholinergic innervation to the hippocampus, and atrophy beginning at approximately 4–6 mos of age [24–26]. However, we did not find a decrease in hippocampal ChAT intensity between young and old Ts65Dn mice, suggesting that there is either an increase in ChAT protein production per neuron and/or an increase in cholinergic fiber branching, resulting in preserved elevated levels of ChAT intensity within the hippocampus throughout middle adulthood ages. It is possible that an increase in ChAT intensity with aging, similar to that found in 2N mice, is masked by age-related BFCN phenotypic degeneration in Ts65Dn mice.

4.2. Genotype Differences

In this study, immunohistochemical and biochemical analyses revealed a subregion-specific elevation of ChAT intensity within the hippocampus at 14–18 mos and an overall elevation of ChAT activity in unsupplemented Ts65Dn compared with unsupplemented 2N mice at 14–18 mos. Previous studies have reported similar higher levels of ChAT-positive fiber innervation and ChAT enzyme activity in the hippocampus and neocortex of Ts65Dn mice compared with 2N mice at 10–12 mos [20,21]. In previous studies, measurement of the activity of the ACh degrading enzyme AChE revealed an upregulation in the olfactory cortex but not in hippocampus in 10- and 19-month-old Ts65Dn mice [20]. Together with the present study, these findings suggest a disconnect within the hippocampal cholinergic biochemistry in the Ts65Dn mouse. Whether cholinergic biochemical function in the forebrain cholinergic system is hindered by increased ChAT fiber reorganization, or whether this increased innervation is indicative of biochemical and structural alterations remains unknown.

Although we observed genotype-dependent differences in levels of hippocampal ChAT intensity, no differences were found in the overall pattern of cholinergic innervation. Unlike neuron number, fiber branching is generally considered a dynamic, neuroplastic process. Neuronal processes fluctuate in accordance with a multitude of factors: diet, insult, disease, hormones, age, external experience, internal computation, and even circadian rhythms, among others [88–93]. The fact that we found elevations in both young [67] and older (present study) mice suggests a homeostatic drive upon forebrain cholinergic tone associated with the aneuploidic state.

In rodent dentate gyrus, spines are primarily located on the outer two-thirds of dendrites in the hippocampal molecular layer, a primary site for glutamatergic input [94]. Increased cholinergic innervation to the IML at the expense of innervation to the OML would likely disrupt excitatory input, interfering with spatial summation of glutamatergic input to the

hippocampus. Since we did not find evidence that such remodeling occurs in Ts65Dn mice, it is likely that the behavioral decrements reported in prior studies result from other types of neuronal dysfunction [37]. Moreover, increased innervation alone may be indicative of dysfunction resulting in the inability to discriminate between task-relevant and task-irrelevant signals [64]. More work is needed to determine whether the differences reported here have direct behavioral correlates, or whether there are compensatory alterations at cholinergic sites that prevent detectable differences at the behavioral level.

4.3. Maternal Choline Supplementation

MCS increased ChAT intensity in the hippocampus and DG of adult Ts65Dn mice, similar to our previous findings showing an increase in hippocampal ChAT intensity in MCS Ts65Dn mice at 4–6 mos of age [67]. The consistent cholinergic elevation across the animal's lifespan suggests that MCS produces an organizational upregulation of cholinergic innervation within the hippocampus beginning early in development. Although few previous studies have been conducted using prenatal choline-supplemented disomic rodents over 12 mos of age, a single study reported increased ACh evoked-release in the hippocampus of prenatal choline-supplemented aged rats (> 24 mos) compared to unsupplemented controls [95]. Due to choline's known role in epigenetic remodeling [96,97], particularly within the cholinergic system [98], MCS may have a profound, lifelong cellular effect through methylation that is beneficial to the offspring independent of genotype [96–99].

4.4. Functional Consequences of Increased Hippocampal Cholinergic Innervation Associated with Aging, Genotype, and MCS

Correlational analyses provided insight into the functional consequences of increased hippocampal ChAT intensity seen with aging, trisomy, and MCS. A significant positive association was seen between number of maze errors and degree of ChAT intensity in the hippocampus of unsupplemented mice of both genotypes. Increased cholinergic tone may reflect an attempt to compensate for a progressively dysfunctional system, similar to what is observed in human prodromal AD where ChAT activity increases in the forebrain before ultimately decreasing as dementia progresses [83–87,99]. However, the association between poorer performance suggests a maladaptive compensatory response, similar to that reported following temporal lobe epilepsy [100,101].

In contrast, a strong negative correlation between number of maze errors and hippocampal ChAT intensity was seen in the supplemented 2N mice, demonstrating that increased ChAT immunoreactivity associated with MCS is beneficial depending on genotype. This suggests the MCS-related increased cholinergic tone observed in 2N mice is fundamentally different from that observed in Ts65Dn mice or during aging of 2N mice. No correlation between errors and ChAT intensity was found in the supplemented Ts65Dn mice; however, as these mice show elevated innervation throughout life, we might be observing a plateau for behavioral impact.

Understanding the specific mechanism(s) by which MCS exerts life-long effects on offspring hippocampal cholinergic innervation is an area of great interest. Organizational brain changes may result from choline's role as the precursor to phosphatidylcholine, a

major constituent of neuronal cellular membranes, and, to a lesser extent, as a precursor of ACh, which is a key ontogenetic signal during development [34,51,57,102]. Additionally, long-term effects of early choline supplementation may be related to epigenetic factors with lasting effects on gene expression, secondary to choline's role as a methyl donor [39,103–105].

Despite a lack of understanding the mechanism(s) of action of MCS, several rodent studies have shown that supplementing the maternal diet with additional choline produces lifelong beneficial cognitive effects for offspring and reduces age-related cognitive decline [48,106,107]. MCS also ameliorates dysfunction in rodent models of human disorders including fetal alcohol syndrome, Rett syndrome, epilepsy, and schizophrenia [58,60,62,63,108]. Since nerve growth factor (NGF) is involved in the maintenance of BFCNs during development and in DS models of axonal transport whereby NGF is inhibited, it is important to consider interaction(s) between neurotrophic activity and choline supplementation as well as hippocampal neuroplasticity and behavioral responses in Ts65Dn and 2N mice [26,31]. The present findings suggest that increasing maternal choline intake during pregnancy provides, in part, some developmental and age-related protection towards the hippocampal cholinergic system dysfunction seen in DS

CONCLUSIONS

The findings reported here demonstrate that increased hippocampal ChAT immunoreactive intensity occurs with aging in 2N mice, while Ts65Dn mice have elevated innervation compared with 2N mice at multiple ages but do not show a significant aging effect. Interestingly, increased cholinergic tone correlated with poorer cognitive performance in Ts65Dn and 2N mice born to unsupplemented Ts65Dn dams, while an opposite pattern was seen in 2N mice that received MCS. The genotype-dependent differential mechanisms impacting behavior remain elusive, but we hypothesize that the changes reflect an attempt at neural reorganization, that may or may not be beneficial to the animal depending on genotype. Although increases in ChAT activity similar to those observed in the present study have been seen during the early phase of AD, these increases did not co-occur with structural changes in cholinergic fiber innervation [89], suggesting that the aging brain has the capacity to mount a neuroplastic biochemical response in the face of neurodegenerative insults. Such a response may be muted in a developmental disorder, such as DS.

Nonetheless, prior studies from our group have shown that enhancing the septohippocampal cholinergic system from an early age provides long-lasting benefit on the cognitive deficits observed in DS [37]. Our structure-function studies suggest that a simple dietary treatment approach such as early choline supplementation may prove a partial prophylaxis for cholinergic related deficits in AD of disomic and DS populations. Overall, MCS appears to be a promising treatment approach for neurodevelopmental disorders including DS.

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Figure 1. Average ChAT intensity in the hippocampus and dentate gyrus of male Ts65Dn and 2N mice at 6–8 and 14–18 mos

(A) Ts65Dn mice had overall higher levels of cholinergic innervation than 2N mice, as measured by ChAT-immunolabeling intensity, and 2N mice showed a significant increase with age (* $p < 0.05$, ‡ $p < 0.001$, Mann-Whitney U, two-tail, $n = 10-13$) Values are plotted as reciprocal (x^{-1}) of luminosity and were derived by averaging measurements in all laminae and across the three sections along the rostrocaudal access used for evaluation, shown in B. Data presented represent medians for group.

Figure 2. Average ChAT-positive fiber innervation in the hippocampus of MCS (+) and unsupplemented (–) male Ts65Dn and 2N mice at 14–18 mos

ChAT intensity averaged across all regions and laminae is shown as medians for each group and location. Ts65Dn supplemented mice had consistently higher values of ChAT intensity than 2N supplemented mice, significant in the caudal section (* $p < 0.05$, Mann-Whitney U, two-tail, $n = 11$ for each group). Values are plotted as reciprocal (x^{-1}) of luminosity measures.

Figure 3. Cholinergic innervation to the hippocampus proper laminae in MCS (+) and unsupplemented (-) male Ts65Dn mice and disomic littermates (2N) at 14–18 mos

Line plots traversing the rostral through caudal hippocampus with measurements taken at laminae of the CA region were drawn using group medians for measurements at the *stratum oriens* (s.o.), *stratum pyramidale* (s.pyr.), *stratum radiatum* (s.r.), and *stratum lacunosum moleculare* (s.lm.). **(Left panel)** Similar patterns of innervation were observed between groups in all hippocampal laminae (* $p < 0.05$, ** $p < 0.01$; 1, 2N-compared with Ts65Dn-; 4, 2N+ compared with Ts65Dn+, Mann-Whitney U, two-tail, $n = 11$ per group). **(Right panel)** ChAT intensity was consistently higher in CA3 than CA1, (significance $p < 0.05$ or greater for all groups, not shown, Friedman test). All values plotted are reciprocal (x^{-1}) values of luminosity measurements with light-microscope photomicrographs. CA1, *cornu ammonis* 1; CA3, *cornu ammonis* 3.

Figure 4. Cholinergic innervation to the dentate gyrus in MCS (+) and unsupplemented (-) male Ts65Dn mice and disomic littermates (2N) at 14–18 mos

Line plots depict median ChAT luminosity measurements from the rostral hippocampus through the caudal hippocampus, with measurements taken at the hilus (hil), granule layer low density (g1), granule layer high density (g2), and dentate gyrus inner molecular layer (iml) and outer molecular layer (oml). (**Left panel**) Laminae-dependent alterations to the dentate gyrus were observed with maternal choline supplementation (* $p < 0.05$, ** $p < 0.01$; 1, 2N- compared with Ts65Dn-; 2, 2N- compared with 2N+, 4, 2N+ compared with Ts65Dn+, Mann-Whitney U, two-tail, $n = 11$ per group), and (**Right panel**) comparison of the inner molecular layer (IML) with the outer molecular layer (OML) shows consistently higher innervation to the OML for all groups (* $p < 0.05$, ** $p < 0.01$, † $p < 0.001$, Friedman test, two-tail, $n = 11$ per group). All values plotted represent group medians and are reciprocal (x^{-1}) values of luminosity measurements with light-microscope photomicrographs.

Figure 5. Cholinergic innervation to the molecular layers of the dentate gyrus in MCS (+) and unsupplemented (-) male Ts65Dn mice and disomic littermates (2N) at 14–18 mos
Elevated ChAT intensity is seen in the inner molecular layer (IML) of Ts65Dn mice compared with 2N mice independent of treatment condition, but only in the outer molecular layer (OML) for groups that received MCS (* p < 0.05, ** p < 0.01; Mann-Whitney U, two-tail, n = 11 per group). All values represent medians and are plotted as reciprocal (x⁻¹) values of luminosity measurements derived using light-microscope photomicrographs.

Figure 6. Correlations between behavioral performance on the RAWM and ChAT intensity levels in the hippocampus

Mean of errors in representative block 3 of the hidden platform task positively correlated with the intensity of ChAT immunolabeling in the inner molecular layer (IML) and outer molecular layer (OML) of the dentate gyrus as well as in the hippocampus proper for maternal choline supplemented (+) and unsupplemented (–) male Ts65Dn (Ts) mice and disomic (2N) littermates at 14–18 mos. r_s , Spearman's rank correlation coefficient

Figure 7. Hippocampal ChAT enzyme activity in MCS (+) and unsupplemented (–) male Ts65Dn mice and disomic littermates (2N) at 15–19 mos

ChAT activity is elevated in the hippocampus of Ts65Dn mice compared with age-matched 2N mice, independent of maternal diet treatment group ($\dagger p < 0.005$, $\ddagger p < 0.001$; Mann-Whitney U, two-tail, $n = 15$ – 18 per group). Values plotted are representative of group medians.

Table 1**Animal groups**

Aging Cohort	n^a	Age range (mean)
Young-adult mice (6–8 mos)		
2N	13	6.5–8.0 (7.7) mos
Ts65Dn	10	6.5–8.1 (7.5) mos
Late middle-aged mice (14–18 mos)		
2N	13	14.0–17.9 (16.4) mos
Ts65Dn	11	13.9–18.4 (16.3) mos
Hippocampal ChAT Intensity and Behavioral Analysis Cohort		
n^a Age range (mean)		
2N		
Control diet	11	13.7–17.8 (15.6) mos
Maternal choline-supplemented	11	13.7–17.6 (15.4) mos
Ts65Dn		
Control diet	11	13.7–17.4 (15.9) mos
Maternal choline-supplemented	11	13.8–17.6 (15.4) mos
Hippocampal ChAT Activity Cohort		
n^a Age range (mean)		
2N		
Control diet	15	14.7–17.3 (16.3) mos
Maternal choline-supplemented	18	15.7–18.4 (16.8) mos
Ts65Dn		
Control diet	15	15.2–17.9 (16.5) mos
Maternal choline-supplemented	18	16.2–18.6 (17.3) mos

^a Group sizes

Table 2

ChAT intensity in the hippocampus proper and dentate gyrus across aging

	Hippocampus proper							
	CA3/2				CA1			
	s.o.	s.pyr.	s.rad.	s.o.	s.pyr.	s.rad.	gran.	dentate gyrus iml oml
<u>Rostral</u>								
2N 6-8 mos	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Ts65Dn 6-8 mos	1.05*	1.08*	1.05	1.04*	1.06*	1.04	1.04**	1.08** 1.06**
2N 14-18 mos	1.05**	1.09**	1.03	1.01	1.04*	1.02	1.04*	1.06* 1.06*
Ts65Dn 14-18 mos	1.06	1.10	1.04	1.05	1.06	1.05	1.05	1.10 1.10
<u>Middle</u>								
2N 6-8 mos	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Ts65Dn 6-8 mos	1.04**	1.05	1.03	1.05	1.08*	1.04	1.05**	1.10** 1.08**
2N 14-18 mos	1.00	1.03	1.00	0.99	1.02	0.98	1.03	1.03* 1.04*
Ts65Dn 14-18 mos	1.03	1.06	1.02	1.02	1.03	1.00	1.04	1.07 1.07
<u>Caudal</u>								
2N 6-8 mos	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Ts65Dn 6-8 mos	1.00	1.00	1.00	1.04**	1.04	1.01	1.02	1.03 1.02
2N 14-18 mos	1.00	1.03	0.99	0.99	1.01	0.98	1.02	1.01 1.02
Ts65Dn 14-18 mos	1.02	1.04	1.01	1.03	1.05	1.01	1.05	1.07 1.06

* p < 0.05,

** p < 0.01 compared with 2N at 6-8mos;

Mann-Whitney U, n = 10-13. No measure in Ts65Dn mice at 14-18 mos was significantly different from Ts65Dn mice at 6-8 mos. CA1, cornu ammonis 1; CA3/2, cornu ammonis 3/2; iml, inner molecular layer; gran, granule layer; oml, outer molecular layer; s.o., stratum oriens; s.pyr., stratum pyramidale; s.rad., stratum radiatum

Table 3

Effects of genotype and maternal choline supplementation on ChAT innervation in the hippocampus of Ts65Dn and 2N mice

	Genotype		Diet	
	2N Control Diet vs Supp Diet	Ts65Dn Control Diet vs Supp Diet	Ts65Dn vs 2N Control Diet	Ts65Dn vs 2N Choline Supp Diet
Rostral				
CA1 s.o.	— ^a	—	—	—
CA1 s.pyr.	—	—	—	—
CA1 s.rad.	—	—	—	—
CA s.l.m.	—	↑4%	↑4%	↑8%*
CA3/2 s.o.	↑4%	↑4%	↑3%	↑3%
CA3/2 s.pyr.	↑4%	↑5%	↑3%	—
CA3/2 s.rad.	—	—	—	—
DG granule	↑4%*	↑6%	↑6%	↑7%*
DG iml	—	↑4%	↑7%*	↑5%**
DG oml	↑4%	—	↑8%	↑5%*
Middle				
CA1 s.o.	—	—	—	—
CA1 s.pyr.	—	—	—	—
CA1 s.rad.	—	—	—	—
CA s.l.m.	—	—	↑4%	↑5%
CA3/2 s.o.	—	—	—	—
CA3/2 s.pyr.	—	—	—	↑3%
CA3/2 s.rad.	—	↑3%	—	↑4%
DG granule	—	↑8%	↑3%	↑14%*
DG iml	—	↑3%	↑8%**	↑10%*
DG oml	—	—	↑7%	↑7%*
Caudal				
CA1 s.o.	—	—	—	—
CA1 s.pyr.	—	—	—	—
CA1 s.rad.	—	—	—	—
CA s.l.m.	—	—	↑3%*	↑4%*
CA3/2 s.o.	—	—	↑6%	↑5%
CA3/2 s.pyr.	—	—	—	↑3%
CA3/2 s.rad.	—	—	—	—
DG granule	—	↑3%	↑5%*	↑7%**
DG iml	—	—	↑9%*	↑8%**

	Genotype		Diet	
	2N Control Diet vs Supp Diet	Ts65Dn Control Diet vs Supp Diet	Ts65Dn vs 2N Control Diet	Ts65Dn vs 2N Choline Supp Diet
DG oml	—	—	↑9%	↑8%*

^a decrease 3 % (↓), increase 3 % (↑), or change < |3| % (—);

* p < 0.05,

** p < 0.01.

CA1, *cornu ammonis* 1; CA3/2, *cornu ammonis* 3/2; DG, dentate gyrus; iml, inner molecular layer; oml, outer molecular layer; s.l.m., stratum lacunosum moleculare; s.o., stratum oriens; s.pyr., stratum pyramidale; s.rad., stratum radiatum.