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Maternal choline supplementation differentially alters the basal forebrain cholinergic system of young-adult Ts65Dn and disomic mice

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Abstract

Down syndrome (DS), trisomy 21, is a multifaceted condition marked by intellectual disability and early presentation of Alzheimer's disease (AD) neuropathological lesions including degeneration of the basal forebrain cholinergic neuron (BFCN) system. While DS is diagnosable during gestation, there is no treatment option for expectant mothers or DS individuals. Using the Ts65Dn mouse model of DS that displays age-related degeneration of the BFCN system, we investigated the effects of maternal choline supplementation on the BFCN system in adult Ts65Dn mice and disomic (2N) littermates at 4.3–7.5 mos of age. Ts65Dn dams were maintained on a choline supplemented diet (5.1 g/kg choline chloride) or a control, unsupplemented diet with adequate amounts of choline (1 g/kg choline chloride) from conception until weaning of offspring; postweaning, offspring were fed the control diet. Mice were transcardially perfused with paraformaldehyde, brains were sectioned, and immunolabeled for choline acetyltransferase (ChAT) or p75-neurotrophin receptor (p75^{NTR}). BFCN number and size, the area of the regions, and the intensity of hippocampal labeling were determined. Ts65Dn unsupplemented mice displayed region- and immunolabel-dependent increased BFCN number, larger areas, smaller BFCNs, and overall increased hippocampal ChAT intensity compared with 2N unsupplemented mice. These effects were partially normalized by maternal choline supplementation. Taken together, the results suggest a developmental imbalance in the Ts65Dn BFCN system. Early maternal-diet choline supplementation attenuates some of the genotype-dependent alterations in the BFCN system, suggesting this naturally occurring nutrient as a treatment option for pregnant mothers with knowledge that their offspring is trisomy 21.

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Conflict of Interest Statement

There are no conflicts of interest.

Role of Authors

Study concept and design: BJS, EJM, SDG, CMK. Acquisition, analysis, and interpretation of data: CMK. Statistical analysis: CMK. Drafting of manuscript: CMK. Critical revision of the manuscript: CMK, EJM, SDG, BJS. Obtained funding: BJS, EJM, SDG. Administrative, technical, and material support: BEP, JAA, RV.

Keywords

Choline acetyltransferase; diagonal band; Down syndrome; medial septum; nucleus basalis; p75 neurotrophin receptor

Introduction

Down syndrome (DS) is a multi-faceted condition caused by triplication of human chromosome 21 (HSA21) that results in alterations of cardiac, respiratory, endocrine, gastrointestinal, and immunological systems. In addition to physiological health concerns, individuals with DS present with cognitive impairment in the domains of learning, memory, and language, and have increased prevalence of select neuropsychiatric conditions (Hayes and Batshaw, 1993; Myers and Pueschel, 1991). While systemic alterations, health complications, and IQ range vary throughout the DS population, intellectual disability (ID) is considered a hallmark of DS (Määttä, et al., 2006; Epstein, 1995). Compounding ID, is a premature aging phenotype that includes neuropathologic features similar to Alzheimer's disease (AD) presenting by age forty and a clinical presentation of dementia in ~50% of individuals over the age of fifty (Wegiel, et al., 1996; Hof, et al., 1995; Wisniewski, et al., 1985; Mann, et al., 1984).

Currently, specific neuropathological causes of ID are unknown; although, degeneration of the basal forebrain cholinergic neuron (BFCN) system is a major pathological feature of both AD and DS, manifesting as decreased BFCN size, density, and ultimately partial cell loss accompanied by altered innervation and neurotrophic profiles in cholinceptive cortical and limbic projection sites (Sendera, et al., 2000; Jørgensen, et al., 1990; Mufson, et al., 1989; Coyle, et al., 1988; Casanova, et al., 1985; Yates, et al., 1983, 1980). This neuropathology is mirrored in the Ts65Dn mouse model of DS and AD (Contestabile, et al., 2006; Seo and Isacson, 2005; Cataldo, et al., 2003; Hunter, et al., 2003; Cooper, et al., 2001; Granholm, et al., 2000; Holtzman, et al., 1996). Ts65Dn mice have segmental trisomy of a region of murine chromosome 16 (MMU16) that contains genes orthologous to HSA21 (Kahlem, et al., 2004; Gardiner, et al., 2003; Akesson, et al., 2001; Reeves, et al., 1995). Several of the triplicated genes are relevant to neurodegenerative conditions, including AD, for example amyloid-beta precursor protein (APP), superoxide dismutase 1 (SOD1), and dual-specificity tyrosine-phosphorylation regulated kinase 1a (DYRK1A); additionally, triplicated HSA21 genes can influence expression and activity of disomic genes, for example through the action of transcription factors and kinases/phosphatases (Vilardell, et al., 2011; Wegiel, et al., 2011; Liang, et al., 2008; Shankar, et al., 2007; Lesné, et al., 2006; Margallo-Lana, et al., 2004; Walsh, et al., 2002; Prasher, et al., 1998).

Although there are no effective treatment strategies for ID, DS, or AD neuropathology and dementia, a promising treatment approach for ID and DS is maternal supplementation with choline. Choline is an essential nutrient which requires intake through the diet in addition to physiological synthesis. In the developing fetus, choline is involved in effective neural tube closure, organogenesis, central nervous system cell membrane synthesis, and control of gene expression through DNA methylation (Wurtman, et al., 2009; Niculescu, et al., 2004; Cooney, et al., 2002; Fisher, et al., 2002, 2001; Zeisel, 2000; Blusztajn, 1998; Zeisel and Blusztajn, 1994). Maternal choline supplementation has both immediate and long-term beneficial effects in normal, disomic rats and mice (Li, et al., 2004; Mellott, et al., 2004; Sandstrom, et al., 2002; Meck, et al., 2003, 1999, 1997, 1989, 1988; Tees, 1999; Pyapali, et al., 1998; Holler, et al., 1996; Loy, et al., 1991) and in rodent models of prenatal ethanol exposure, Rett syndrome, and status epilepticus (Thomas, et al., 2009, 2007; Nag, et al., 2008; Ward, et al., 2008; Nag and Berger-Sweeney, 2007; Holmes, et al., 2002).

Unfortunately, reports suggest that pregnant and nursing women consume lower amounts of choline than recommended by the National Academy of Sciences and Institute of Medicine (Jensen et al., 2007; Gossell-Williams, et al., 2005; Shaw, et al., 2004). While this does not appear to have overt effects in a normal, disomic population (Signore, et al., 2008), the consequences for a trisomic population may be detrimental, particularly in relation to the development and maintenance of the BFCN system.

BFCNs in the medial septum/vertical limb of the diagonal band (MS/VDB) and nucleus basalis of Meynert (NBM) are integral anatomic substrates subserving memory and attention through their cholinergic input to the hippocampus and cortex, respectively (Hasselmo and Sarter, 2011; Rye, et al., 1984; Mesulam et al., 1983). Disruption of these systems may underlie some of the cognitive deficits seen in ID, DS, and AD. To explore the effects of early choline supplementation upon the BFCN projection system, prior to the onset of age-related degeneration, we used a maternal supplementation paradigm, followed by assessment of the segmentally trisomic Ts65Dn mice and disomic (2N) littermates at young-adult ages. Several independent groups have reported early atrophic changes in Ts65Dn mice, beginning as early as 2 mos of age, including decreased BFCN size, endosomal abnormalities, and defective retrograde transport; however, BFCN loss, phenotypic silencing, and decreased hippocampal cholinergic innervation are most consistently reported after 10 mos of age (Contestabile, et al., 2006; Seo and Isacson, 2005; Cataldo, et al., 2003; Hunter, et al., 2003; Cooper, et al., 2001; Granholm, et al., 2000; Holtzman, et al., 1996). In the present study, we determined the effects of maternal choline supplementation on the BFCN system in Ts65Dn mice at 4.3–7.5 mos. Ts65Dn mice and 2N littermates were exposed to either a choline-supplemented or unsupplemented, choline-controlled treatment condition via the maternal diet, from conception to weaning. Currently, data on maternal choline supplementation in the Ts65Dn mouse is limited. In this regard, our group has shown improvements on attention tasks in older Ts65Dn mice born to choline-supplemented dams (Moon, et al., 2010) and we recently demonstrated that maternal choline supplementation increases adult hippocampal neurogenesis and improves performance on the radial-arm water maze in adult Ts65Dn mice (Velazquez, et al., 2013). The long-term effects of early choline supplementation on the BFCN system in Ts65Dn mice and 2N littermates have not yet been explored.

Materials and Methods

Subjects

Female Ts65Dn and male C57BL/6J*EiJ*×B6*EiC3SnF1/J* mice were obtained from Jackson Laboratories (Bar Harbor, Maine) and bred at Cornell University (Ithaca, NY). This murine line carries a gene for retinal deterioration (*rd*), *Pde6b*^{rd1}, which presents in a Mendelian recessive manner and causes blindness (Holtzman, et al., 1996; Keeler, 1966). Mice homozygous *rd/rd* were excluded from analysis, resulting in 25 Ts65Dn offspring and 24 2N offspring between the ages of 4.3–7.5 mos (mean age = 5.9 mos; Table 1) available for analysis. Jackson Laboratories performed genotyping using qRT-PCR from tail snip or ear punch samples (Liu, et al., 2003). All procedures were approved by the Institutional Animal Care and Use Committee of Cornell University and conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Choline supplementation

One-half of the dams received a diet supplemented with choline (5.1 g/kg choline chloride in AIN-76A, Dyets, Inc., Bethlehem, PA, USA) beginning at the time of breeding and continuing until weaning, similar to that reported previously (Velazquez, et al., 2013). Non-supplemented dams received a choline-controlled diet containing the amount of choline in

standard rodent chow (1.1 g/kg choline chloride in AIN-76A, Dyets, Inc.). Offspring born to dams maintained on the control diet are referred to as “unsupplemented” although it should be noted this does not refer to a deficient treatment condition. Both maternally choline-supplemented and unsupplemented offspring remained with their original dams until weaning. At weaning, mice were housed in same-sex, mixed-genotype groups and maintained on the control, unsupplemented diet (1.1 g/kg, Dyets, Inc.). Fourteen Ts65Dn and 12 2N mice received choline supplementation (Table 1).

Tissue preparation

All mice were deeply anesthetized by intraperitoneal injection of a ketamine:xylazine (90mg/kg:9.5mg/kg) solution, then transcardially perfused with ice-cold saline (0.9 % NaCl), followed by 4 % paraformaldehyde, as described previously (Kelley, et al., 2013). Brains were removed from the calvarium and placed in the same fixative at 4° C for 24 h, then immersed in a sucrose solution (30 %) until sectioning on a freezing sliding microtome in the coronal plane at 40-µm thickness into 6 series. Sections were stored at 4° C in cryoprotectant (30 % glycerol, 30 % ethylene glycol, 40 % phosphate buffer) until processing.

Immunohistochemistry

Immunohistochemistry was performed as described previously (Kelley, et al., 2013, 2011; Overk, et al., 2009). A full series of sections from each animal was singly immunolabeled with either a goat polyclonal antibody for choline acetyltransferase (ChAT, 1:1000 dilution, Millipore, Billerica, MA, USA) (Raghanti, et al., 2008), the non-rate-limiting enzyme for acetylcholine synthesis and a specific marker for cholinergic neurons that specifically recognizes cholinergic neurons (Mufson, et al., 1989; Rye, et al., 1984; Levey, et al., 1983), or a rabbit polyclonal antibody against the 75 kDa pan-neurotrophin receptor p75^{NTR} (1:2000, Millipore), a receptor involved in a multitude of functions, including cell survival and death, that colocalizes with BFCNs (Koh and Loy, 1989; Mufson et al., 1989). Briefly, tissue was washed in phosphate buffer (PB) to remove excess cryoprotectant, rinsed in Tris-buffered saline (TBS), and incubated in sodium *metaperiodate* to inhibit endogenous peroxidase activity. To enhance primary antibody penetrance, tissue was washed in TBS containing 0.25 % Triton X-100 followed by incubation in a blocking solution consisting of 3% serum in TBS/Triton X-100 (ChAT, horse serum; p75^{NTR}, goat serum). Tissue was then incubated overnight at room temperature with either ChAT or p75^{NTR} primary antibodies in a solution of TBS/Triton X-100 with 1 % serum. All washes and incubations were carried out at room temperature on a shaker table.

Following overnight incubation in primary antibody, sections were washed in TBS and incubated for 1 h with a biotinylated secondary antibody (ChAT, anti-goat IgG, host horse; p75^{NTR}, anti-rabbit IgG, host goat; Vector Laboratories, Inc., Burlingame, CA, USA). Sections were washed in TBS, then, to amplify the signal, sections were incubated for 1 h in avidin-biotin-complex solution (Elite kit, Vector Laboratories, Inc.). Tissue was then washed in a sodium imidazole acetate buffer, and antibody immunolabeling for ChAT and p75^{NTR} was visualized using a chromogen solution consisting of 0.05 % 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich, St. Louis, MO, USA), 1 % nickel (II) ammonium sulfate hexahydrate, and 0.0015 % H₂O₂, resulting in a black reaction product (Figs. 1, 2, 3). The DAB/Ni-peroxide reaction was terminated with a series of washes in fresh imidazole-acetate buffer solution; sections were then rinsed in PB, mounted on chrome-alum-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 two consecutive sets, one for each immunolabel, with animals randomized

across baskets to reduce batch-to-batch variation and variation in group staining. Immunohistochemical controls were conducted on tissue processed at the same time as ChAT and p75^{NTR} immunolabeling with the omission of primary antibodies. All procedures were performed blind to genotype and treatment.

Antibody characterization

For immunolabeling of BFCNs, we used an affinity-purified goat polyclonal antibody raised against human placental ChAT enzyme (Millipore, catalog number AB144P; Table 2), and a Protein A affinity-purified rabbit polyclonal antibody raised against a glutathione S-transferase (GST) fusion protein that corresponds to residues 274–425, within the intracellular domain of rat p75^{NTR} (Millipore, catalog number 07–476). Antibody specificity for ChAT is confirmed with human placental and rat forebrain lysates and is routinely evaluated by western blot analysis conducted on mouse brain lysates (<http://www.millipore.com/catalogue/item/ab144p>, Retrieved 2013). Antibody specificity for p75^{NTR} is confirmed with rat forebrain sections and is routinely evaluated by western blot analysis conducted with radio immunoprecipitation assay (RIPA) buffer lysates from pheochromocytoma line 12 (PC-12) cells (<http://www.millipore.com/catalogue/item/07-476#>, Retrieved 2013). The staining pattern in the present study was consistent with cholinergic neuron localization throughout the forebrain and brainstem for both markers (Figs. 1, 2, 3). **Antibody tissue penetration**

A major requirement for using the optical disector probe with the optical fractionator is that antibodies penetrate the full depth of immunolabeled sections, allowing for equal probability of counting objects throughout the section depth, one of the prerequisites for unbiased stereology. We determined full antibody penetration throughout tissue thickness by examining marker placement in the z-axis after each optical fractionator run. Markers were placed only when a planar surface of the cell was fully in focus and post-run analysis revealed all sections demonstrated similar patterns of marker z-depth, including equal placement at the z-axis tissue center.

Basal forebrain regions

BFCN regions were defined according to a previous publication (Mufson et al., 1989). Three subregions were examined: the MS/VDB, horizontal limb of the diagonal band (HDB), and NBM/substantia innominata (NBM/SI). In preliminary studies we saw no difference between MS and VDB measures, so these regions were combined. Cholinergic fiber innervation within the hippocampus proper and dentate gyrus was examined at three points along the rostrocaudal axis (Fig. 6C, D, E). Hippocampal laminae were defined according Paxinos and Franklin (2001).

Morphometric analysis

All morphometric measures were performed using Stereo Investigator software (version 9.14.5 32-bit, MicroBrightField, Inc., Williston, VT, USA) coupled to a Nikon Optiphot-2 microscope, as described previously (Kelley, et al., 2013). To control for inter-individual variability, a single investigator performed all analyses. To ensure low intra-individual variability, five mice chosen at random were measured non-consecutively three times during each triad (beginning, middle, end) of experiment. No significant difference was found across these time points. All analyses were conducted blinded to genotype and treatment.

Photomicrographs were taken on a Nikon Optiphot-2 microscope (Tokyo, Japan) connected to Stereo Investigator software (MicroBrightField, Inc.) (Figs. 1, 2, 3, 6). Background correction was used at the time of image capture to establish evenness of illumination across the field, and scale bars were added within the Stereo Investigator software. Panels were

compiled in PowerPoint (version 14.0.6129.5000, 32-bit, Microsoft, Redmond, WA, USA), and in Figs. 1, 2, and 3 each micrograph was equally corrected for brightness and contrast. No retouching or further manipulations were performed.

Cell count and density

ChAT- and p75^{NTR}-positive neuron counts were derived with the optical fractionator, a stereological probe that allows for unbiased sampling throughout the region of interest. Values are presented as estimate per brain, derived from a bilateral sampling of 11 % of the region area across a 1/6 series for each immunolabel (X60 oil-immersion lens, numerical aperture (n.a.) 1.40, counting frame 50 × 50 μm, grid size 151 × 151 μm, disector height 8 μm, estimate derived from the reciprocal for [(disector height)/(mean measured thickness)]) in accord with contemporary methods and theory (Stereology Resource Center, 2008; Glasser, et al., 2007; Mouton, 2002). The large sampling fraction allowed for a CE, $m = 1$, of 0.10 (Gundersen, et al., 1999). Cell density is presented as cells per 1,000,000 μm³ and was derived by dividing the total marker count by number of sampling sites with adjustment to represent a 1,000,000⁻¹ sampling fraction. Calculating density in this manner avoids the additional variability that is introduced when cell counts (counted within the three-dimensional optical disector space) are divided by a calculated region volume (derived by region area which is measured across a two-dimensional plane). All calculations were performed for each animal, prior to group averages.

Area measurement

The area of basal forebrain subregions (μm²) was derived by planimetry and the values presented represent summation of contours in one series drawn around the region of interest (one-sixth the brain; X1 lens, n.a. 0.04; MS/VDB, average of 5 sections; HDB, 4 sections; NBM/SI, 6 sections). Measurements were taken and averaged separately for each immunolabeled series. Previous comparison between Cavalieri estimator and planimetry values for 27 region measurements showed no difference between methods (slope $m = 1.001$, correlation $r^2 = 0.998$, $r = 0.999$). A relative area was calculated by averaging measures for ChAT- and p75^{NTR}-immunolabeled tissue and summing all three basal forebrain subregions, calculated within subject prior to deriving group averages, and values are presented relative to 2N unsupplemented mice.

Cell size

Cell size was measured using a 5-ray nucleator probe for 50 cells per animal (X60 oil-immersion lens, n.a. 1.40; Stereo Investigator, MicroBrightField, Inc.) using random sampling across rostrocaudal and dorsoventral axes, as derived using the optical fractionator (Gundersen, 1988). The 5-ray nucleator probe involves five measurements from an approximate center of the cell to the perimeter of the cell taken in one plane of section (<1.0 μm z-axis). An average radius is then derived for each cell and area and volume are calculated from this value using classic planimetry and geometry formulae with shape

assumption circular or spherical respectively: $area = \pi(length)^2$ and $volume = \frac{4\pi}{3}(length)^3$, wherein *length* is the mean average of five radial measurements.

Immunolabeling intensity measurements in the hippocampus and dentate gyrus

The intensity of ChAT immunolabeling was determined by tracing the hippocampus and dentate gyrus unilaterally at three points along the rostrocaudal axis using an X1 lens (n.a. 0.04) (Fig. 6C, D, E). 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. 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 immunolabeling was measured at 23 sites (Fig. 6B) using ImageJ software (1.45s, 1.6.0_20, 32-bit; Rasband, 1997–2012). As seen in Fig. 6B, these included nine sites in the dentate gyrus, eleven sites across the hippocampus proper, and three background sites within the corpus callosal white matter situated above the hippocampus (not shown). The three background measurements were averaged per section, and each hippocampal ChAT intensity measurement was divided by the average background measurement. No difference was seen between the ventral and dorsal blades of the dentate gyrus (Fig. 6B), so the data were averaged. Total hippocampal ChAT intensity shown in Fig. 6A was derived by averaging all measurements across the dentate gyrus and hippocampus proper, and ChAT intensity in the hippocampus proper shown in Fig. 7A was derived by averaging CA2/3, CA1/2, and CA1 regions (Fig. 6B). 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).

Statistical analysis

The nonparametric Mann-Whitney U test was used for determining differences between groups, and the Friedman test was used for within-group comparisons. Nonparametric statistics were used owing to unequal variances between groups. Because the tests involve analysis of ranks, and median is a more accurate descriptor of group averages, all values are presented as median. Data from male mice fell within the first through third interquartile ranges of data from female animals, so male and female mice were pooled for all measures. Statistics were conducted using Excel (version 14.0.6129.5000, Microsoft) and SPSS (PASW Statistics 18, release 18.0.0, IBM, Armonk, NY, USA). Statistical significance was set at $p < 0.05$ in nondirectional two-tailed tests.

Results

Region-dependent alterations in p75^{NTR}-positive BFCNs in Ts65Dn mice

Quantitative analysis showed significantly more p75^{NTR}-positive BFCNs (by 24 %), and a nonsignificant higher number of ChAT-positive BFCNs (by 6 %) in the MS/VDB of unsupplemented Ts65Dn mice compared with unsupplemented 2N littermates (p75^{NTR} $p < 0.01$, ChAT $p = 0.16$, Fig. 1Q, Table 3). Similarly, unsupplemented Ts65Dn mice had 36 % more p75^{NTR}-positive BFCNs in the HDB than unsupplemented 2N mice, although the elevation was not significant ($p = 0.12$), while no difference was seen for ChAT-positive BFCN number between genotypes in the unsupplemented treatment condition (Fig. 2Q, Table 3). Maternal choline supplementation attenuated the elevation observed in MS/VDB p75^{NTR}-positive BFCNs of Ts65Dn mice ($p = 0.05$) without altering the number of MS/VDB p75^{NTR}-positive BFCNs in 2N mice or ChAT-positive BFCNs in either genotype (Fig. 1Q, Tables 3 and 4). In contrast, maternal choline supplementation decreased ChAT- and p75^{NTR}-positive BFCN counts in the HDB of Ts65Dn and 2N mice (Ts65Dn, ChAT $p = 0.06$, p75^{NTR} $p < 0.05$; 2N, ChAT $p = 0.09$, p75^{NTR} $p > 0.20$; Fig. 2Q, Tables 3 and 4). We found a nonsignificant 16 % lower number of p75^{NTR}-positive cells in the NBM/SI of unsupplemented Ts65Dn mice compared to unsupplemented 2N littermates (Fig. 3Q, Table 3) and comparable numbers of ChAT-positive BFCNs in these groups. Maternal choline supplementation did not significantly alter NBM/SI ChAT- or p75^{NTR}-positive cell counts for either genotype.

Larger area in Ts65Dn mice

We observed a significant average 17 % larger MS/VDB area in unsupplemented Ts65Dn compared with unsupplemented 2N mice (ChAT and p75^{NTR} $p < 0.01$, Fig. 4A, Table 3), and a nonsignificant average 12 % larger HDB area of unsupplemented Ts65Dn mice compared with unsupplemented 2N mice (Fig. 4B, Table 3). MS/VDB area was unchanged by maternal choline supplementation (Fig. 4A, Table 3); while, maternal choline supplementation led to a significant average 20 % decrease in HDB area in Ts65Dn mice (ChAT and p75^{NTR} $p < 0.05$; Fig. 4B, Table 3). In these regions, no significant change was observed in 2N mice that received choline supplementation compared with unsupplemented 2N mice (Fig. 4A, B, Table 4). Area of the NBM/SI was comparable between genotypes and treatment conditions (Fig. 4C, Tables 3 and 4).

With areas for the separate subregions combined (see Methods), we found a significant 16% larger overall basal forebrain size in Ts65Dn unsupplemented mice compared with 2N unsupplemented littermates ($p < 0.01$, Fig. 4D). The larger size was partially attenuated in Ts65Dn mice that received choline supplementation, resulting in a nonsignificant 5 % larger area in Ts65Dn supplemented compared to 2N supplemented mice (Fig. 4D).

Maternal choline supplementation normalizes Ts65Dn alterations in BFCN density

For unsupplemented animals, a nonsignificant average 12 % higher density for ChAT- and p75^{NTR}-positive BFCNs was found in Ts65Dn compared with 2N mice for the MS/VDB (Fig. 1R), and a significant 32 % higher density for ChAT-positive BFCNs ($p < 0.05$) and nonsignificant 19 % higher density for p75^{NTR}-positive BFCNs in the HDB (Fig. 2R, Table 3). Maternal choline supplementation did not significantly affect ChAT-positive BFCN densities for either genotype in the MS/VDB. However, a significant 14 % decrease in ChAT-positive BFCN density was seen in the HDB of Ts65Dn supplemented compared with Ts65Dn unsupplemented mice ($p < 0.05$), lowering the level closer to that seen in 2N mice of either treatment condition (Fig. 2R, Table 3). Similar decreases in Ts65Dn supplemented compared with Ts65Dn unsupplemented mice were observed in the MS/VDB and HDB for p75^{NTR}-immunolabeled tissue, although neither region reached significance (MS/VDB, 15 % decrease, $p = 0.11$; HDB, 15 % decrease, $p = 0.16$; Figs. 1R, 2R, Table 3). In contrast, ChAT- and p75^{NTR}-immunolabeled BFCN densities were comparable in the NBM/SI independent of genotype or treatment (Fig. 3R, Tables 3 and 4).

Hypotrophy of ChAT- and p75^{NTR}-positive BFCNs in Ts65Dn mice

Quantitative analysis across the three regions revealed that ChAT-positive BFCNs were on average 15 % smaller in Ts65Dn unsupplemented compared with 2N unsupplemented mice (MS/VDB $p > 0.20$, HDB $p < 0.05$, NBM/SI $p < 0.01$, Fig. 5, Table 3). A similar hypotrophy was observed for p75^{NTR}-positive cells in the HDB, where a significant 16 % smaller p75^{NTR}-positive BFCN size was seen in Ts65Dn unsupplemented mice compared with 2N unsupplemented mice ($p < 0.05$, Fig. 5B, Table 3). However, the size of p75^{NTR}-positive cells was comparable across genotypes in the MS/VDB and NBM/SI of unsupplemented animals (Fig. 5A, C, Table 3).

Maternal choline supplementation resulted in an average 19 % decrease in ChAT-positive BFCN size in 2N mice across all subregions (MS/VDB $p < 0.01$, HDB $p > 0.20$, NBM/SI $p < 0.01$, Fig. 5, Table 4). A similar effect was not found for p75^{NTR}-immunolabeled cells, where p75^{NTR}-positive BFCN size was comparable between 2N maternally choline supplemented and unsupplemented mice. In Ts65Dn mice, no effect from maternal choline supplementation was observed on BFCN size for either marker in the MS/VDB or HDB (Fig. 5A, B, Table 3). However, in the NBM/SI, a significant average 18 % increase in both ChAT- and p75^{NTR}-positive BFCN size was observed in maternally choline supplemented

Ts65Dn compared with unsupplemented Ts65Dn mice ($p < 0.05$, Fig. 5C, Table 3). Importantly, there was no correlation between cell density and cell size in any subregion (data not shown, MS/VDB and HDB $r = 0.006$, NBM/SI $r = 0.160$).

Hippocampal Cholinergic Innervation

Overall, the intensity of ChAT immunolabeling was elevated in the hippocampus proper and dentate gyrus of Ts65Dn unsupplemented compared with 2N unsupplemented mice (Fig. 6A), and these levels remained higher in Ts65Dn mice that received maternal choline supplementation, with no difference observed between Ts65Dn maternally choline supplemented and unsupplemented mice (Fig. 6A). In contrast, 2N mice that received maternal choline supplementation showed increases in ChAT-positive fiber innervation compared with 2N unsupplemented mice (caudal $p < 0.05$, Fig. 6A).

Laminar hippocampal ChAT intensity

Analysis of ChAT-positive fiber innervation within laminae CA1-3 of the hippocampus (Fig. 6B), revealed no difference within stratum oriens (SO) or stratum pyramidale (SPyr) in the rostral hippocampus between Ts65Dn and 2N unsupplemented mice (Fig. 7A). We observed elevated levels of ChAT-positive innervation within the stratum lacunosum moleculare (SLM, Fig. 6B) of Ts65Dn compared with treatment-matched 2N mice in the middle and caudal sections of the hippocampus (unsupplemented, middle and caudal $p < 0.05$; supplemented, middle $p < 0.01$, caudal not significant.; Fig. 7A, C).

Maternal choline supplementation resulted in decreased ChAT-positive innervation in the rostral SPyr of 2N and Ts65Dn mice, when compared with genotype-matched unsupplemented mice (2N $p < 0.05$, Ts65Dn $p = 0.08$, Fig. 7A). Contrastingly, increased ChAT-positive fiber innervation was seen with maternal choline supplementation in the SLM in middle and caudal hippocampal sections for 2N supplemented compared with 2N unsupplemented mice (middle $p = 0.06$, caudal $p < 0.05$), with no significant change observed in Ts65Dn mice (Fig. 7C).

Overall, ChAT-positive fiber innervation was greater in CA3 than CA1, independent of genotype or treatment condition (Fig. 7B), although patterns of lamination were similar across all groups with the highest ChAT intensity values observed in the SPyr and the lowest values in the stratum radiatum (SRad) of CA1 and CA3. Increased innervation with maternal choline supplementation was observed in CA3 of the middle and caudal sections for 2N mice and CA3 of the middle section for Ts65Dn mice, compared with genotype-matched unsupplemented mice (Fig. 7B). This effect was only observed for CA1 in the middle section of 2N mice (Fig. 7B). As with total hippocampal innervation, ChAT-positive fiber innervation was elevated in CA1 of Ts65Dn unsupplemented and supplemented mice compared with 2N unsupplemented mice (Figs. 6A, 7B).

Dentate gyrus ChAT intensity

Unsupplemented Ts65Dn mice had significantly higher levels of ChAT intensity when compared with unsupplemented 2N mice in both the inner molecular layer (IML) and outer molecular layer (OML) of the caudal dentate gyrus ($p < 0.05$, Fig. 7D, E, G). Maternal choline supplementation resulted in significantly increased levels of ChAT intensity in the OML of 2N mice ($p < 0.05$, caudal section) and a nonsignificant increase in the IML ($p = 0.08$, caudal section; Fig. 7D, F, G). This effect was not mirrored in Ts65Dn mice, where the high levels of ChAT-positive fiber innervation seen in unsupplemented Ts65Dn mice were maintained in mice that received maternal choline supplementation (Fig. 7F, G).

Within-group comparisons revealed comparable levels of ChAT-positive fiber innervation between the IML and OML of all groups within the rostral dentate gyrus (Fig. 7D, E). In contrast, a nonsignificant average 23 % increase in ChAT intensity was observed in the IML, compared to the OML, of Ts65Dn unsupplemented mice in the middle and caudal sections (middle $p = 0.10$, caudal $p = 0.06$, Fig. 7E), and an average 11 % increase in ChAT intensity in the IML, compared to the OML, of Ts65Dn maternally choline-supplemented mice in the middle section ($p < 0.05$, Fig. 7E). ChAT intensity levels were comparable between the IML and OML of rostral and middle sections in 2N mice of either treatment condition. Whereas, a significant 17 % increase in ChAT intensity was seen in OML, compared to the IML, in the caudal section of 2N unsupplemented mice ($p < 0.05$) and a nonsignificant 24 % increase in the OML, compared to the IML, in 2N supplemented mice ($p = 0.10$, Fig. 7E).

Discussion

Genotype Differences Between Ts65Dn and 2N mice

Although assessment of the BFCN system in Ts65Dn mice has been performed to varying degrees by several independent research groups, a systematic, unbiased morphometric evaluation of BFCNs throughout multiple basal forebrain subregions and corresponding assessment of cholinergic fiber innervation within hippocampal laminae has been lacking. Further, no biological study has been conducted on the potential ameliorative effects of maternal choline supplementation in Ts65Dn mice. The effects of maternal choline supplementation of the BFCN system in Ts65Dn and 2N mice observed here are summarized in Tables 3 and 4. We found a significantly higher number of p75^{NTR}-positive BFCNs in the MS/VDB of young-adult (4.3–7.5 mos) Ts65Dn unsupplemented mice compared with 2N unsupplemented mice, with no change in neuron density. In contrast, previous reports did not find a significant difference in p75^{NTR}-positive BFCN number at 6 mos in the MS between Ts65Dn and 2N mice fed a standard diet (Cooper, et al., 2001; Holtzman, et al., 1996). The different results may be related to group size variation between studies, with the current investigation examining 11–14 subjects per condition, versus prior studies that examined only 5–6 mice per group. The results are likely not due to differences in region of analysis, as preliminary investigation did not show a difference between the MS and VDB in the present study. We found no difference between genotypes for MS/VDB ChAT-positive BFCN number or density of unsupplemented mice, supporting other reports showing no significant difference in ChAT-positive BFCN density in the MS or VDB between Ts65Dn and 2N mice at 5–8 and 6–10 mos of age (Kelley, et al., 2013; Granholm, et al., 2000).

We also found an increase in the area of this region in tissue stained for p75^{NTR} in Ts65Dn unsupplemented mice compared with 2N unsupplemented littermates. In contrast, only one other study evaluated area in this murine line and found no difference between genotypes for MS or VDB in ChAT-immunolabeled tissue at ages 4 and 8 mos (Granholm, et al., 2000). Together these findings suggest that cellular machinery within BFCNs relating to neurotrophic and neural transmission are differentially affected by genotype. Moreover, area alterations suggest an underlying genotype-dependent developmental hypertrophy in Ts65Dn mice that is not responsive to maternal choline supplementation.

Examination of cell size revealed hypotrophy of ChAT-positive neurons in the MS/VDB, HDB, and NBM/SI, and selectively for p75^{NTR}-positive HDB neurons in Ts65Dn unsupplemented compared with 2N unsupplemented mice. Previous reports observed similar hypotrophy of BFCNs immunolabeled for ChAT, p75^{NTR}, or TrkA in the MS and VDB of Ts65Dn compared with 2N mice from 6–10 mos of age, and in aged mice from 18–22 mos (Lockrow, et al., 2011, 2009; Salehi, et al., 2006; Cooper, et al., 2001; Granholm, et al.,

2000; Holtzman, et al., 1996). Opposing results are observed for mice at the mid-adult ages, with no difference in the size of ChAT-positive cells seen between genotypes within the MS or with the MS/VDB/HDB combined, at 11–14 mos (Seo and Isacson, 2005; Granholm, et al., 2002). In contrast to ChAT, we did not observe significant differences in the size of p75^{NTR}-positive cells of the MS/VDB between Ts65Dn and 2N mice. These findings suggest that in addition to being genotype-dependent, phenotype and age affect BFCN size. Currently, there are no comparable reports for the HDB or NBM/SI at this age.. Notably, smaller MS cells visualized using a Nissl stain were observed in Ts65Dn compared with 2N mice at 12–15 mos (Chen, et al., 2009), suggesting further morphometric and chemoarchitectonic studies are needed to determine whether cellular hypotrophy is specific to BFCNs. Hypotrophy of Ts65Dn BFCNs may represent an early growth retardation that differentiates Ts65Dn from 2N mice, and where amenable to a therapeutic intervention, such as in the NBM/SI with maternal choline supplementation, a plausible treatment model with translational potential to DS humans exists.

Quantitative assessment of ChAT-positive fiber innervation within the hippocampus and dentate gyrus revealed higher ChAT intensity levels in all laminae within young adult Ts65Dn unsupplemented compared to 2N unsupplemented mice. Previous studies found similar increases in hippocampal ChAT innervation and ChAT activity in Ts65Dn compared with 2N mice at 10–12 mos as well as in other BFCN projections sites such as the olfactory bulb and neocortex (Contestabile, et al., 2006; Seo and Isacson, 2005). Independent research groups have reported similar regional and laminar alterations in cholinergic staining intensity in the hippocampus and cingulate cortex using the indirect cholinergic marker acetylcholinesterase (AChE), including increased labeling in the CA3 region compared with other hippocampal subfields as well as increased p75^{NTR}-immunolabeled fiber intensity in the IML compared to the OML in Ts65Dn mice (Chen, et al., 2009; Salehi, et al., 2006). In the present investigation, higher levels of hippocampal cholinergic innervation were not associated with a greater number of ChAT-positive MS/VDB neurons between genotypes. However, these higher levels correlated with a significant increase in p75^{NTR}-positive MS/VDB neurons in Ts65Dn unsupplemented compared with 2N unsupplemented mice. The findings suggest a potential cholinergic neuroplastic response within the hippocampus that is independent of alterations in BFCN number, but likely coincides with underlying differences in phenotypic expression, possibly similar to cholinergic upregulation in the hippocampus of individuals with MCI (Ginsberg, et al., 2006; Counts and Mufson, 2005; Ikonomic, et al., 2003; DeKosky, et al., 2002; Davis, et al., 1999; Bierer, et al., 1995), and if the elevated cholinergic innervation in the hippocampus of Ts65Dn mice is a disease related phenomenon, the increased innervation may represent a compensatory response.

Alternately, the elevated cholinergic fiber intensity within the hippocampus in Ts65Dn mice at this young-adult age suggests upregulation of ChAT protein or enzyme activity levels within the MS/VDB neurons during development. Whether these changes are compensatory for other developmental deficits, such as cholinergic receptor or transport system alterations, remains to be determined. One consideration is that reactive synaptogenesis may be occurring, such as has been demonstrated for cholinergic terminals in the rodent hippocampus (Steward et al., 1988; Nadler, et al., 1977). Future studies could aim to test whether Ts65Dn mice demonstrate altered patterns of reactive synaptogenesis or lesion-induced plasticity (Ginsberg, 2005; Cotman, et al., 1977), and the degree to which any detrimental consequences of these compensations are amenable to maternal choline supplementation.

Maternal Choline Supplementation Partially Attenuates Neuropathology in Ts65Dn mice

Maternal choline supplementation resulted in decreased numbers of p75^{NTR}-positive MS/VDB and HDB neurons in Ts65Dn mice, regions where elevations were seen in

unsupplemented Ts65Dn mice over unsupplemented 2N mice. In the NBM/SI, maternal choline supplementation resulted in significantly larger ChAT- and p75^{NTR}-positive neurons in Ts65Dn mice, a region displaying BFCN hypotrophy in unsupplemented Ts65Dn compared with unsupplemented 2N mice. These data suggest that maternal choline supplementation partially normalizes the genotype-dependent alterations in cell number and size observed in unsupplemented mice. Choline has a primary role as a methyl donor, through the betaine-methionine pathway, and alterations in dietary levels of choline, folate, and methionine can affect gene expression (Pogribny, et al., 2008; Kovacheva, et al., 2007; Niculescu, et al., 2004; Waterland and Jirtle, 2003; Cooney, et al., 2002). Alterations in epigenetic programming may be one possible mechanism for lasting phenotypic alterations seen following early intervention with maternal choline supplementation.

To date, no studies have examined BFCN numbers in maternally choline-supplemented Ts65Dn or 2N mice and no comparable studies for supplemented disomic rodents of other strains. However, one study reported no change in BFCN counts for the MS, VDB/HDB, and NBM in 8–9-month rats subjected to prenatal choline deficiency (McKeon-O'Malley, et al., 2003). Whether alterations in prenatal choline intake induce phenotypic changes of select BFCN markers is unknown. Our data, which shows a significant decrement of p75^{NTR}-positive BFCNs in the HDB with parallel but nonsignificant changes seen for ChAT-positive HDB neurons, supports the possibility of a region-specific downregulation of BFCNs in Ts65Dn mice that is independent of phenotypic markers. Based on studies that have shown the optimal environment for the adult animal is one that mirrors the prenatal environment (Wong-Goodrich, et al., 2008), it would be interesting to examine whether decrements in p75^{NTR}-positive BFCNs can be rescued by adult choline supplementation in Ts65Dn maternally supplemented mice.

Maternal choline supplementation resulted in smaller ChAT-positive BFCNs in all regions of 2N supplemented compared with 2N unsupplemented mice, but did not alter p75^{NTR}-positive cell size in 2N mice. These results contrast with a prior study in rats that found an increase in p75^{NTR}-positive cell size in the MS/VDB of pre- and postnatally choline-supplemented rats at 6–7 mos (Williams, et al., 1998), although several methodological differences between studies make direct comparisons difficult; these include differences in: group sizes, operational definition and sampling of cells, method of choline supplementation, and the species examined. Notably, 2N controls in our study were born to, and reared by, Ts65Dn dams. Whether neurodevelopmental organizational differences from an altered hormonal milieu, epigenetic influences that occur during conception and *in utero* development, or Ts65Dn maternal rearing impact 2N development is largely unknown. It has been shown that Ts65Dn dams take poorer care of their offspring (Salehi, et al., 2009; Roper, et al., 2006), the long-term effects of which have not been fully explored. While the 2N littermates utilized in this study are well suited for comparisons with their trisomic siblings, the data generated may not be directly comparable to studies using other strains of disomic rodents.

The current investigation revealed that maternal choline supplementation resulted in increased ChAT-positive fiber innervation within the hippocampal formation of 2N mice. This is in contrast to Ts65Dn mice, where ChAT-positive fiber intensity was consistently high in the hippocampus of unsupplemented and supplemented Ts65Dn mice, when compared with unsupplemented 2N mice. A review of the literature failed to reveal studies of ChAT-immunolabeling intensity in adult disomic animals or Ts65Dn mice that received early choline supplementation. However, early maternal choline supplementation in rats produces decreases in ChAT, AChE, and high-affinity choline uptake activity levels in the hippocampus during the first two months of life (Cermak et al., 1999, 1998; Blusztajn, et al., 1998), as well as increased evoked ACh release (Meck, et al., 2007; Cermak, et al., 1998). It

is unclear whether these changes persist throughout adulthood and into old age. Some have reported no significant alterations at 3 mos (Cermak, et al., 1999), while others found increased evoked release of ACh persisting in rats over 24 mos of age (Meck, et al., 2007). The importance of increased cholinergic fiber levels in the hippocampus seen in the present study following maternal choline supplementation in 2N mice remains a topic for future investigation. The potential benefit of increased cholinergic tone may represent a neuroplasticity response that could be exploited as a therapeutic approach for translation to AD as well, providing that the mechanism(s) underlying this response are elucidated. Interestingly, patients with mild AD given a nutrient drink containing, among other factors, choline showed improved memory on a delayed verbal recall task (Scheltens, et al., 2010). The extent to which supplementing the diet with naturally occurring elements can ameliorate symptoms and, with an adequate early biomarker, delay onset of neurodegenerative conditions is still in the early stages of study, but evidence is mounting for their therapeutic potential, especially if susceptible populations can be determined and treated early enough, such as is the case with DS.

Therapeutic Implications

While many genes triplicated in DS are implicated in ID and age-related BFCN pathology, a specific metabolic pathway responsible for the pathological conditions seen in DS humans or trisomic murine models remains unknown. Consequently, it is difficult to develop an effective treatment approach for this disorder. Treatment of DS children and nondemented DS adults with the AChE inhibitor, donepezil, has shown mixed results on tests of cognition, with some finding improvements on select language tasks, but overall no impact on ID or adaptive behavior problems (Kondoh, et al., 2011; Kishnani, et al., 2009; Spiridigliozzi, et al., 2007; Heller, et al., 2004; Johnson, et al., 2003). Another clinical trial with donepezil proved ineffective in DS adults with signs of dementia (Prasher, et al., 2002); although, a small study with a convenience sample found some benefit with this drug (Lott, et al., 2002). In addition, a recent randomized, double-blind placebo-controlled clinical trial in DS patients over 40 yrs of age with dementia given the FDA AD-approved drug memantine, an extrasynaptic NMDA receptor antagonist, found no clinical benefit (Hanney, et al., 2012), despite earlier studies demonstrating improvements in learning in Ts65Dn mice treated with memantine (Mohan, et al., 2009; Costa, et al., 2008). There are conflicting reports on whether the cholinergic system of DS humans is affected from birth (Becker, et al., 1991; Kish, et al., 1989; Casanova, et al., 1985; McGeer, et al., 1985), postnatally, or during the aging process. The role the BFCN system plays in DS as well as when (and how) to begin treatment is not evident at this time due to an incomplete understanding of cholinergic dysfunction in the human disorder and in relevant animal models.

In the present study, Ts65Dn and 2N mice received choline supplementation from conception to weaning. The extended period was chosen based upon previous reports demonstrating embryonic and postnatal sensitive periods for choline supplementation in healthy disomic animals and in animals with fetal alcohol exposure or Rett syndrome (Thomas, et al., 2009, 2007; Nag, et al., 2008; Meck, et al., 2007; Nag and Berger-Sweeney, 2007; Williams, et al., 1998) as well as the fact that development of the BFCN system continues postnatally (Berger-Sweeney, et al., 2001; Brady, et al., 1989; Semba and Fibiger, 1988; Armstrong, et al., 1987). Moreover, choline supplementation from conception to weaning has lasting benefits on attention, emotional reactivity, and hippocampal neurogenesis in adult Ts65Dn mice (Moon, et al., 2010; Velazquez, et al., 2013). The amount of choline supplementation provided to our mice is comparable to that used in investigations conducted over the past three decades (Mellott, et al., 2004; Li, et al., 2004; Sandstrom, et al., 2002; Meck, et al., 1999, 1997, 1989, 1988; Tees, 1999; Pyapali, et al., 1998; Holler, et al., 1996; Schenk and Brander, 1995; Loy, et al., 1991) and translates to

values within the normal range of human consumption, well below doses considered to have negative side effects (Detopoulou, et al., 2008; National Academy of Sciences and Institute of Medicine, 1998). The normalization of BFCN number in the MS/VDB and HDB and of BFCN size in the NBM/SI we observed in Ts65Dn mice who received maternal choline supplementation as well as positive effects upon cholinergic fiber innervation within discrete hippocampal laminae of 2N mice with supplementation suggests this may be a viable treatment strategy for life-long improvement of the cognitive functions mediated by BFCN system neuromodulation in DS, and possibly beneficial for delayed onset and slowed rate of decline in AD. In summary, phenotypic assessment of BFCN size, number, and basal forebrain area, in addition to evaluation of cholinergic efferents to the hippocampal formation (and possibly other targets such as the olfactory complex and neocortex) appears to be a viable readout to assess the potential beneficial effects of treatment modalities such as maternal choline supplementation on animal models of DS. Coupled with coordinated behavioral, epigenetic, cellular, and molecular assessments, this approach may yield mechanistic data that translates to rational therapeutic intervention for the amelioration of ID and/or neuropathology associated with DS and AD.

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Figure 1.

Photomicrographs show the distribution of (A–H) ChAT and (I–P) p75^{NTR} immunoreactivity within the MS/VDB of 6-month 2N and Ts65Dn mice. Mice were exposed to either an unsupplemented, choline-controlled (–) or a choline-supplemented (+) treatment via maternal diet from conception to weaning. Scale bars in D and L = 100 μm and in H and P = 25 μm, and apply to all panels in the corresponding row. Bar graphs show (Q) significantly higher number of p75^{NTR}-immunolabeled cells in the MS/VDB of Ts65Dn mice exposed to an unsupplemented, choline-controlled condition (–) from conception to weaning compared with unsupplemented 2N mice (** p < 0.01, Mann-Whitney U, two-tail,

n = 11–14), and **(R)** BFCN density is comparable across genotypes and treatments in the MS/VDB.

Figure 2.

Photomicrographs show the distribution of (A–H) ChAT and (I–P) p75^{NTR} immunoreactivity within the HDB of 6-month 2N and Ts65Dn mice. Mice were exposed to either an unsupplemented, choline-controlled (–) or a choline-supplemented (+) treatment via maternal diet from conception to weaning. Scale bars in D and L = 100 μm and in H and P = 25 μm and apply to all panels in the corresponding row. Bar graphs show (Q) maternal choline supplementation significantly decreased the number of p75^{NTR}-immunoreactive cells in the HDB of Ts65Dn mice (* *p* < 0.05, Mann-Whitney U, two-tail), and (R) BFCN density is but elevated in unsupplemented Ts65Dn mice compared with unsupplemented 2N littermates in the HDB, and decreased in the HDB of Ts65Dn mice who received maternal choline supplementation. (* *p* < 0.05, Mann-Whitney U, two-tail, *n* = 11–14)

Figure 3.

Photomicrographs show the distribution of (A–H) ChAT and (I–P) p75^{NTR} immunoreactivity within the NBM/SI of 6-month 2N and Ts65Dn mice. Mice were exposed to either an unsupplemented, choline-controlled (–) or a choline-supplemented (+) treatment via maternal diet from conception to weaning. Scale bars in D and L = 100 μm and in H and P = 25 μm, and apply to all panels in the corresponding row. Bar graphs show BFCN (Q) count and (R) density is comparable across genotypes and treatments in the NBM/SI.

Figure 4.

Bar graphs show differences in regional area for ChAT- and p75^{NTR}-immunolabeled basal forebrain cholinergic subfields in 2N and Ts65Dn mice given unsupplemented (unSUPP) or choline supplemented (choline-supp) diets from conception to weaning. (A) Increased area is seen for the MS/VDB in ChAT- and p75^{NTR}-immunolabeled tissue (* $p < 0.05$, ** $p < 0.01$, Mann-Whitney U, two-tail, $n = 11-14$) independent of treatment condition in Ts65Dn mice, and (B) region area is decreased with maternal choline supplementation in the HDB of Ts65Dn mice. (C) No difference between genotypes or treatment conditions is observed in NBM/SI area. (D) Relative basal forebrain area shows an increase in Ts65Dn over 2N mice. In panels A, B, C scale on left ordinate axes are for ChAT and p75^{NTR} (gray and black bars) and scale on right ordinate axes are for average values (white bars).

Figure 5.

Bar graphs show alteration in BFCN cell size. (A) Decreased ChAT-positive cell size with maternal choline supplementation (+) was seen in the MS/VDB of 2N mice, and (B, C) hypotrophy of Ts65Dn ChAT- and p75^{NTR}-positive BFCNs was observed in the unsupplemented treatment condition (-) in the HDB and NBM/SI. * p < 0.05, ** p < 0.01, Mann-Whitney U, two-tail, n = 11–14.

Figure 6.

(A) Intensity of ChAT-immunoreactivity was increased in Ts65Dn mice independent of treatment condition but only in 2N littermates who received maternal choline supplementation from conception to weaning (+) in the mid and caudal hippocampus (*2N- x 2N+ $p < 0.05$ 2N- compared with 2N+; Mann-Whitney U, two-tail, $n = 11-14$). (B) A representative section shows hippocampal laminae analyzed: CA1, cornu ammonis 1; CA1/2, cornu ammonis 1/2; CA2/3, cornu ammonis 2/3; _d, dorsal; iml, inner molecular layer; g1, granule layer, high density; g2, granule layer low density; hil, hilus; oml, outer molecular layer; s.lm., stratum lacunosum moleculare; s.o., stratum oriens; s.pyr., stratum pyramidale; s.r., stratum radiatum; _v, ventral. (C-E) Low magnification photomicrographs demonstrate the three sections used for (C) rostral, (D) middle, and (E) caudal analysis. Scale bars = 500 μm.

Figure 7.

(A) Graphic representation showing differences between genotype and treatment determined by averaging the CA2/3, CA1/2, and CA1 ChAT intensity levels (see Fig. 6B–E for subregion map and sections of analysis, * $p < 0.05$, ** $p < 0.01$, ¹ 2N- compared with Ts-, ² 2N- compared with 2N+, ³ 2N+ compared with Ts+, Mann-Whitney U, two-tail, $n = 11-14$). (B) Graphic representation showing a higher density of ChAT staining in CA2/3 than CA1. (C) A significant increase in ChAT intensity was found in the mid s.lm. of Ts65Dn compared to 2N mice independent of treatment, and in the caudal s.lm. of unsupplemented Ts65Dn mice. An increase in ChAT intensity with maternal choline supplementation was seen in 2N mice in the caudal s.lm. (D) Cholinergic innervation within the dentate gyrus shows differential distribution across layers, (E) with increased innervation is seen in the IML compared to the OML of Ts65Dn mice, and in the OML compared to the IML in 2N mice (* $p < 0.05$, Friedman test, two-tail, $n = 11-14$). (F–G) Increased innervation in Ts65Dn mice compared to 2N mice is observed in both the IML and OML. Values plotted are reciprocal (x^{-1}) values of luminosity measurements determined with light-microscope photomicrographs.

Table 1

Animal groups

	n^a	Age range (mean)
Disomic		
Unsupplemented, normal diet	12 (9F, 3M)	4.5 – 7.5 (5.8) mos
Perinatal choline-supplemented	12 (8F, 4M)	4.3 – 7.5 (5.7) mos
Ts65Dn		
Unsupplemented, normal diet	11 (9F, 2M)	4.5 – 7.5 (6.2) mos
Perinatal choline-supplemented	14 (13F, 1M)	4.4 – 7.5 (6.0) mos

^a Group sizes; F, female; M, male

Table 2

Antibody Table

Antigen	Immunogen	Manufacturer (cat. no.), host, type	Dilution used
choline Acetyltransferase(ChAT)	human placental ChAT	Millipore (AB144P), goat, polyclonal	1:1000
p75-neurotrophin receptor (p75 ^{NTR})	glutathione S-transferase (GST) fusion protein corresponding to the intracellular domain, residues 274–425, of rat p75 ^{NTR}	Millipore (07–476), rabbit, polyclonal	1:2000

Table 3
Effects of perinatal choline supplementation on BFCN subregions in Ts65Dn mice

	BFCN Count		Region Area		BFCN Density		BFCN size	
	ChAT	p75 ^{NTR}	ChAT	p75 ^{NTR}	ChAT	p75 ^{NTR}	ChAT	p75 ^{NTR}
MS/VDB								
Ts65Dn unsupp ^a	— ^b	↑24% ^{**}	↑18% ^{**}	↑17% ^{**}	—	↑17%	—	—
Ts65Dn choline supp ^c	—	↓12%	—	—	—	↓15%	—	—
HDB								
Ts65Dn unsupp ^a	↑12%	↑36%	—	↑13%	↑32% [*]	↑19%	↓16% [*]	↓16% [*]
Ts65Dn choline supp ^c	↓27%	↓43% [*]	↓17% [*]	↓22% [*]	↓14% [*]	↓15%	—	—
NBM/SI								
Ts65Dn unsupp ^a	—	↓16%	—	—	—	—	↓18% ^{**}	—
Ts65Dn choline supp ^c	↑12%	—	—	—	—	↓13%	↑19% [*]	↑17% [*]

^a Compared with 2N unsupplemented littermates

^b ↓, decrease >10 %; ↑, increase >10 %; —, difference 10 %;

*

p < 0.05;

**

p < 0.01

^c Compared with Ts65Dn unsupplemented mice

Table 4

Effects of perinatal choline supplementation on BFCN subregions in 2N mice

	BFCN Count		Region Area		BFCN Density		BFCN size	
	ChAT	p75 ^{NTR}	ChAT	p75 ^{NTR}	ChAT	p75 ^{NTR}	ChAT	p75 ^{NTR}
MS/VDB								
2N choline-suppl	— ^a	—	—	—	—	—	↓22% ^{**}	—
HDB								
2N choline-suppl	↓24%	↓38%	—	↓18%	—	—	↓17%	—
NBM/SI								
2N choline-suppl	—	—	—	—	↑12%	—	↓19% ^{**}	—

^a Compared with 2N unsupplemented mice: ↓, decrease >10 %; ↑, increase >10 %; —, difference 10 %;^{**} p < 0.01