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Maternal choline supplementation alters basal forebrain cholinergic neuron gene expression in the Ts65Dn mouse model of Down syndrome

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Abstract

Down syndrome (DS), trisomy 21, is marked by intellectual disability and a premature aging profile including degeneration of the basal forebrain cholinergic neuron (BFCN) projection system, similar to what is seen in Alzheimer's disease (AD). Although data indicate that perinatal maternal choline supplementation (MCS) alters the structure and function of these neurons in the Ts65Dn mouse model of DS and AD (Ts), how MCS affects the molecular profile of vulnerable BFCNs is unknown. We investigated the genetic signature of BFCNs obtained from Ts and disomic (2N) offspring of Ts65Dn dams maintained on a MCS diet (Ts+, 2N+) or a choline-normal diet (ND) from mating until weaning, then maintained on ND until 4.4–7.5 months of age. Brains were then collected and prepared for choline acetyltransferase (ChAT) immunohistochemistry and laser capture microdissection followed by RNA extraction and custom-designed microarray analysis. Findings revealed upregulation of select transcripts in classes of genes related to the cytoskeleton (*Tubb4b*), AD (*Cav1*), cell death (*Bcl2*), presynaptic (*Syngr1*), immediate early (*Fosb, Arc*), G protein signaling (*Gabarap, Rgs10*), and cholinergic neurotransmission (*Chrnb3*) in Ts compared to 2N mice, which were normalized with MCS. Moreover, significant downregulation was seen in select transcripts associated with the cytoskeleton (*Dync1h1*), intracellular signaling (*Itpka, Gng3, Mlst8*), and cell death (*Ccng1*) in Ts

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Conflict of Interest Statement

The authors have no conflicts of interest that might be perceived as influencing their objectivity.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

compared to 2N mice that were normalized with MCS. This study provides valuable insight into mechanisms of genotype-dependent differences and the effects of MCS at the molecular level within a key vulnerable cell type in DS and AD.

Keywords

Microarrays; basal forebrain cholinergic neurons; maternal choline supplementation; Down syndrome; laser capture microdissection; Ts65Dn

Introduction

Down syndrome (DS) is a multi-faceted condition caused by triplication of human chromosome 21 (HSA21) that results in altered cardiac, respiratory, endocrine, gastrointestinal, and immunological systems (Bonamico et al., 2001; Cohen, 2006; Dyken, Lin-Dyken, Poulton, Zimmerman, & Sedars, 2003; Fong & Brodeur, 1987; Freeman et al., 2008). In addition to physiological anomalies, individuals with DS present with cognitive deficits in the domains of learning, memory, and language, as well as increased incidence of neuropsychiatric conditions (Alexander et al., 1997; Haxby & Schapiro, 1992; Oliver, Crayton, Holland, Hall, & Bradbury, 1998). While there is significant intra-individual variability in the degree of physiological and cognitive dysfunction, intellectual disability (ID) is considered a hallmark of DS (Epstein, 1995; Maatta, Tervo-Maatta, Taanila, Kaski, & Iivanainen, 2006). Compounding the ID seen in individuals with DS is a premature aging phenotype (Franceschi et al., 2019) that includes a neuropathological profile similar to that seen in Alzheimer's disease (AD), namely, the deposition of amyloid-beta (Aβ) plaques and tau-containing neurofibrillary tangles (NFTs) by early midlife, accompanied by a clinical presentation of dementia in >50 % of individuals over the age of fifty (Dekker et al., 2018; Hof et al., 1995; Mann, Yates, & Marcyniuk, 1984; Perez et al., 2019; Thase, 1982; Wegiel, Wisniewski, Dziewiatkowski, Popovitch, & Tarnawski, 1996; Wisniewski, Dalton, McLachlan, Wen, & Wisniewski, 1985; Wisniewski, Wisniewski, & Wen, 1985).

In both AD and DS, there is age-associated degeneration of basal forebrain cholinergic neurons (BFCNs) (Casanova, Walker, Whitehouse, & Price, 1985; Coyle, Oster-Granite, Reeves, & Gearhart, 1988; Jorgensen, Brooksbank, & Balazs, 1990; Mann et al., 1984; Mufson, Bothwell, & Kordower, 1989; Sendera et al., 2000; Whitehouse et al., 1983; Yates et al., 1983; Yates, Simpson, Maloney, Gordon, & Reid, 1980) located within the medial septum/vertical limb of the diagonal band (MS/VDB), that project to the hippocampus and play a critical role in memory function (Hasselmo & Sarter, 2011; Mesulam, Mufson, Wainer, & Levey, 1983; Rye, Wainer, Mesulam, Mufson, & Saper, 1984). Dysfunction in this cholinergic projection system is also found in the Ts65Dn mouse model of DS making it a valuable translational model to assess cellular and molecular factors underlying degeneration of BFCNs, as well as possible treatment paradigms (Ash et al., 2014; Berger-Sweeney, 2003; Cataldo et al., 2003; Contestabile, Fila, Bartesaghi, Contestabile, & Ciani, 2006; Cooper et al., 2001; Granholm, Sanders, & Crnic, 2000; Holtzman et al., 1996; Hunter et al., 2003; Kelley et al., 2016; Kelley et al., 2014a; Moon et al., 2010; Seo & Isacson, 2005; Strupp et al., 2016).

A potential therapeutic strategy for BFCN degeneration with translational potential is perinatal maternal choline supplementation (MCS). In the developing fetus, choline is involved in effective neural tube closure, organogenesis, central nervous system cell membrane synthesis, and gene expression related to altering DNA methylation (Blusztajn, Cermak, Holler, & Jackson, 1998; Blusztajn, Slack, & Mellott, 2017; Cooney, Dave, & Wolff, 2002; Fisher, Zeisel, Mar, & Sadler, 2001, 2002; Niculescu, Yamamuro, & Zeisel, 2004; Wurtman, Cansev, Sakamoto, & Ulus, 2009; Zeisel, 2000; Zeisel & Blusztajn, 1994). When supplemented *in utero* and in early development, choline has both immediate and long-term beneficial effects in healthy disomic rats and mice (Holler, Cermak, & Blusztajn, 1996; Li et al., 2004; Loy, Heyer, Williams, & Meck, 1991; Meck, Smith, & Williams, 1988, 1989; Meck & Williams, 1997, 1999, 2003; Mellott, Williams, Meck, & Blusztajn, 2004; Pyapali, Turner, Williams, Meck, & Swartzwelder, 1998; Sandstrom, Loy, & Williams, 2002; Tees, 1999) and in rodent models of prenatal ethanol exposure, Rett syndrome, and status epilepticus (Holmes et al., 2002; Nag & Berger-Sweeney, 2007; Nag, Mellott, & Berger-Sweeney, 2008; Thomas, Abou, & Dominguez, 2009; Thomas, Biane, O'Bryan, O'Neill, & Dominguez, 2007; Ward, Agarwal, Wang, Berger-Sweeney, & Kolodny, 2008). Previously, we demonstrated that MCS during pregnancy and lactation attenuates cognitive dysfunction and BFCN degeneration in adult Ts65Dn progeny (Ash et al., 2014; Kelley et al., 2016; Kelley et al., 2014a; Moon et al., 2010; Powers et al., 2017; Strupp et al., 2016). Despite these encouraging findings, the molecular and cellular mechanisms associated with MCS-mediated attenuation of BFCN degeneration remains an under investigated area.

To address this, the present study examined the molecular signature of MS/VDB neurons, identified immunohistochemically using the cholinergic neuron marker choline acetyltransferase (ChAT), and acquired by laser capture microdissection (LCM) in Ts65Dn (Ts) and disomic (2N) mice (Alldred et al., 2018; Mufson et al., 1989). Since the MS/VDB region contains a heterogeneous population of neurons, analyses based on whole region homogenates are ill suited for BFCN-specific profile analysis (Ginsberg, Che, Wuu, Counts, & Mufson, 2006; Ginsberg et al., 2011; Mesulam et al., 1983; Rye et al., 1984). LCM provides a rigorous and reproducible method to isolate single cells based on a specific cellular phenotype (Ginsberg et al., 2018; Ginsberg et al., 2017; Ginsberg, Mufson, et al., 2010) and can be combined with custom-designed microarrays containing probes relevant to BFCNs, DS, cognitive dysfunction, and AD that hybridize the cDNA made from LCM BFCN mRNA isolates (Ginsberg & Che, 2014). This approach is highly effective for comparative group analysis and provides valuable insight into the molecular pathophysiology of DS, AD, and MCS treatment.

Methods

Ts65Dn mouse model

The Ts65Dn DS mouse model has segmental trisomy of murine chromosome Mmu16 and a centromeric low-coding region of Mmu17 imposed by radiation-induced reciprocal translocation (Akeson et al., 2001; Dierssen, Herault, & Estivill, 2009; Duchon et al., 2011; Kahlem et al., 2004; Reeves et al., 1995; Sturgeon & Gardiner, 2011). The probable triplicated section of Mmu17 is not coding sequence (CDS) dense and there were no genes

from the triplicated segment of Mmu17 on our custom-designed array. For clarity, we denote not triplicated using the notation $Mmu16_{NT}$ and $Mmu17_{NT}$.

Subjects

Female Ts65Dn and male *C57BL/6JEiJ×B6EiC3SnF1/J* mice were obtained from Jackson Laboratories (Bar Harbor, Maine, USA) for breeding as described previously (Ash et al., 2014; Kelley et al., 2014a). Use of *C57BL/6JEiJ×B6EiC3SnF1/J* male breeding pairs provided mixed litters of disomic (2N) and segmentally trisomic (Ts) offspring. The males were used for a series of structure-function investigations (Ash et al., 2014; Powers et al., 2017) accounting for the greater number of female mice in the present study. Genotyping was performed via qRT-PCR from tail snip or ear punch samples sent to Jackson Laboratories. Mice homozygous for the autosomal recessive retinal deterioration *Pde6b*^{rd1} gene were excluded from analysis (Holtzman et al., 1995; Keeler, 1966). Animals were bred and maintained at Cornell University (Ithaca, NY, USA). All procedures were approved by the Institutional Animal Care and Use Committee of Cornell University, which conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Maternal choline supplementation (MCS)

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 (offspring, 2N+ and Ts+). Non-supplemented dams received a diet controlled for choline content (1.1 g/kg choline chloride in AIN-76A, Dyets, Inc.), referred to as normal diet (offspring, 2N and Ts). Both MCS and normal diet offspring remained with their original dams until weaning. At weaning mice were housed in same-sex, mixed-genotype groups and maintained on normal diet. At all stages, experimenters were blind to genotype. Groups consisted of 10 mice each (Table 1). The amount of choline supplementation is comparable to that used in other studies (Holler et al., 1996; Li et al., 2004; Loy et al., 1991; Meck et al., 1988; Meck & Williams, 1999; Mellott et al., 2004; Pyapali et al., 1998; Sandstrom et al., 2002; Schenk & Brandner, 1995; Tees, 1999), and translates to values within the normal range of human consumption and the recommended range for pregnant and nursing female humans (Detopoulou, Panagiotakos, Antonopoulou, Pitsavos, & Stefanadis, 2008; Sciences & Medicine, 1998).

Tissue preparation

At 4.4–7.5 mos (Table 1) offspring were deeply anesthetized by intraperitoneal injection of a ketamine:xylazine (90mg/kg:9.5mg/kg) solution and transcardially perfused with 4 % paraformaldehyde. Brains were removed from the calvarium and placed in the same fixative for 24 h, stored at 4° C, followed by transfer into a 30 % sucrose solution until sectioning on a freezing sliding microtome in the coronal plane at 40 μm thickness into 6 series (240 μm between sections), and stored at 4° C in a cryoprotectant solution (30 % glycerol, 30 % ethylene glycol, 40 % phosphate buffer) until immunolabeling.

Immunohistochemistry

ChAT immunohistochemistry was performed as described previously (Kelley, Perez, Overk, Wynick, & Mufson, 2011). A full series of free-floating sections from each animal was washed and incubated in sodium metaperiodate to inhibit endogenous peroxidase activity. Next, tissue was washed in TBS containing 0.25 % Triton X-100, and incubated in a blocking solution consisting of 3 % serum in TBS/Triton X-100 to enhance primary antibody penetrance and block nonspecific binding. Tissue was then incubated overnight with the goat polyclonal antibody for ChAT (1:1000; Millipore, Billerica, MA, USA), 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 (Vector Laboratories, Inc., Burlingame, CA, USA). To amplify the signal, sections were incubated for 1 h in an avidin-biotin-complex solution (Elite kit, Vector Laboratories, Inc.). Before and after 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). ChAT reactivity was visualized with a 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 % H_2O_2 resulting in a black reaction product (Kelley et al., 2014b). Sections were rinsed, mounted on glass slides and dried overnight at room temperature. All procedures were performed blind to genotype and treatment and no gross features disrupted the blinding.

Laser capture microdissection (LCM)

An Arcturus LCM system connected to an inverted microscope with a software-operated stage was used to capture individual ChAT-immunolabeled MS/VDB neurons. The MS/VDB neurons were pooled to ensure that sufficient numbers of ChAT-positive perikarya would be available for analysis. The LCM system captures individual cells through a near-infared laser-charged thermoplastic membrane that adheres tissue excised with an ultraviolet laser. One hundred ChAT-positive MS/VDB neurons per subject were identified with a 1× lens (10× magnification) and cells were outlined with a lasso tool using a 40× lens (magnification 400×) (Mufson et al., 1989). Extracted cholinergic neurons were distributed across the MS/VDB using four slides from a 240 μm series. Throughout the LCM procedure groups were randomized across genotype and treatment.

RNA isolation and cDNA synthesis

RNASE AWAY (Molecular BioProducts, San Diego, CA, USA) was used to clean all surfaces and tools at each step. RNA was isolated using a standard TRIzol-chloroform-isopropanolol protocol. Membranes with LCM isolates were separated from the plastic caps and homogenized by treatment with cold TRIzol Reagent (1 ml, 4° C; Ambion, Carlsbad, CA, USA) at 4° C then put on ice for 5–10 min. For phase separation, chloroform was added to the membrane-TRIzol mixture, vortexed and let sit for 5 min at room temperature. Samples were then centrifuged > 10,000 RPM at 4° C for 20 min. The aqueous RNA phase was extracted and added to the nucleic acid coprecipitant linear acrylamide (Ambion) to aid

in recovery and visualization of pellet at later stages. Isopropanolol was added and mixed by inverting, for RNA precipitation. Samples were then centrifuged (12,000 RPM) at 4° C for 20 min resulting in a visible RNA pellet. Liquid phase was disposed and samples were washed with 80 % EtOH and centrifuged at 12,000 RPM at 4° C for 7 min. Pellets were re-suspended in 24 μl diethyl pyrocarbonate (DEPC)-treated H_2O (Sigma, St. Louis, MO, USA) and treated with an RNASE inhibitor (0.5 μl; SUPERase-In, Ambion).

Terminal continuation (TC) RNA amplification was employed to make dsRNA using methods optimized previously (Alldred, Che, & Ginsberg, 2009). Briefly, a polyT primer was hybridized to the polyA tail of the mRNA by adding 1 μl polyT primer to 6 μl sample and running one cycle (65° 2 min, 45° 1 min). We performed first strand synthesis by adding Superscript III (Invitrogen, Carlsbad, CA, USA), Master Mix (5×FirstStrand, DTT, 10 mM dNTP Invitrogen), RNase Inhibitor (Ambion), TC primers, in DEPC-treated H_2O and running one cycle (50° 60 min, 65° 15 min). Lastly, primers were re-annealed and RNA degraded through adding PCR buffer (Applied Biosystems, Branchburg, New Jersey, USA) and RNaseH (Ambion) in DEPC-treated H_2O and running one cycle (37° 30 min, 95° 3 min, 60° 3 min). cDNA was purified using centrifugal filters (Amicon Ultra 0.5mL 30K NMWL cellulose membrane) as per manufacturer's instructions (Millipore).

Membrane hybridization

Nitrocellulose membranes with embedded probes were used for hybridization of cDNA, as described previously (Alldred et al., 2018). Membranes were stripped in a weak base (0.4N NaOH) in glass tubes placed in a rotating hybridization oven (45 min at 45° C), rinsed (0.2 M Tris pH 7.2, 0.1% SDS (Gibco), 0.1% SSC 20× (Fisher); 1× 1 min, 2× 10 min, 20° C), and incubated in a prehybridization buffer (formamide, Denhardts solution, 20× SSPE (Ambion), 0.1% SDS, 10%, and boiled sheared salmon sperm DNA (Ambion) in DEPC-treated H_2O; 4 h, 42° C). Samples were incubated in a cocktail (10× transcription buffer, DEPC-treated H_2O, 400nM NaCl, dACGTPs (Ambion), DTT, dUTP, RNAse inhibitor), T7 RNA polymerase (Epicentre Biotechnologies, San Diego, CA, USA), and the isotope P^{33} (5 h, 37° C). Samples were added to the membranes and incubated for 18 h at 42° C in a rotating hybridization oven. At all stages, a nylon mesh was used to prevent the membranes from sticking to themselves in the hybridization tubes. A total of 12 membranes were used, batches were processed in groups of 6, and systematic stratified sampling across membranes, batches, and groups was carefully mapped and employed. Two to five membranes were run per animal (average of two per animal).

Membrane hybridization was visualized by transferring the radioactive signal to a phosphor screen placed in a cassette (Amersham Biosciences, Little Chalfont, UK) that applied constant and even pressure for 24 h at room temperature. Images were obtained with a Kodak Digital Science Image Station 440CF (Rochester, NY, USA), and light intensity was quantified with ImageQuant software (Amersham Biosciences).

Nomenclature and chromosome loci

All genes represented by the membrane-embedded probes were re-identified using the Mouse Genome Informatics (MGI) online databases accessed September 2018 (Jackson

Laboratories, Bar Harbor, ME, USA) (Smith et al., 2018) and represent the most recent nomenclature using genome build GRCm38. Gene functional clustering is based on a combination of MGI description and literature relevant to DS and AD.

Membrane normalization and statistical analysis

Post-normalization, the quotients were averaged across repeat arrays, for each gene, within subject. Membrane probes vary in hybridization strength, and were treated separately with each probe as an independent variable. The arrays are a tool for cross-group comparative analysis, rather than precise recordings of expression levels within a subject. Each membrane included negative control probes placed throughout the coordinates. For each gene the signal intensity ratio was modeled as a function of mouse group, using mixed effects models with random mouse effect to account for the correlation between repeated assays on the same mouse (McCulloch, Searle, & Neuhaus, 2008). Significance was set at $p < 0.01$, two-sided; false discovery rate (FDR) based on an empirical null distribution due to strong correlation between genes was controlled at level $q < 0.1$ (Benjamini & Hochberg, 1995; Efron, 2007). There was no association between age and expression levels for any gene ($-0.000 < R^2 < 0.280$).

Nanostring expression verification

For expression verification, NanoString nCounter was performed on select codesets designed in conjunction with NanoString Technologies (Seattle, WA, USA) utilizing 55 genes known to be involved in DS and AD pathology with five internal negative controls and five internal positive control genes. Normalization was performed utilizing the NanoString reference gene normalization protocol (Veldman-Jones et al., 2015). Because of tissue limitation and genes chosen, hippocampal sections were used for verification. This precludes possible confounds from the heterogeneity of MS/VDB neuron subpopulations (Mesulam et al., 1983; Rye et al., 1984). NanoString nCounter statistical analysis was performed on normalized gene expression levels as described previously (Schafer, Dolgalev, Alldred, Heguy, & Ginsberg, 2015). Each gene was modeled as a function of the mouse study group, using mixed effects models with random mouse effect (Alldred, Lee, Petkova, & Ginsberg, 2015a, 2015b; McCulloch et al., 2008), with significance at $\alpha = 0.05$, two-sided.

Results

Differences in total expression profiles

Custom-designed microarray results revealed significant differences in expression profiles across genotypes and treatments (Figs. 1 and 2A). After normalizing to background levels and calculating within-row z scores, we found that the average gene expression for Ts was increased to 1.48-fold 2N mice levels. In treatment groups, average expression level in 2N+ was increased to 1.05-fold compared to 2N, and Ts+ was increased 5.13-fold relative to Ts levels (Fig. 2A). Since comparison of male and female mice showed male expression levels fell within 1.5 × interquartile range of female expression levels we pooled the data (Tukey, 1977).

Expression of triplicated Mmu16 genes

Several genes triplicated in Ts mice have human orthologs implicated in cognitive dysfuntion and AD. The following five triplicated gene transcripts were on the custom-designed microarray: amyloid beta A4 precursor protein (APP, gene *App*), glutamate receptor ionotropic kainate 1 (*Grik1*), superoxide dismutase 1 soluble (*Sod1*), synaptojanin 1 (*Synj1*), and carbonyl reductase 1 (*Cbr1*) (Fig. 2B). Upregulation was found in *Sod1* and *Cbr1* (Fig. 2B). MCS significantly increased expression of *App* in Ts65Dn mice, and *Sod1* and *Cbr1* in 2N mice (Fig. 2B).

Alterations in AD-related transcripts

Of the 42 microarray probes representing AD-associated genes the following were differentially regulated across genotypes: caveolin 1/caveolae protein (*Cav1*), APP-like protein 1 (*Aplp1*) and 2 (*Aplp2*), and serum amyloid P-component (*Apcs*) were significantly upregulated in Ts compared to 2N mice, and nicastrin (*Ncstn*) was significantly downregulated (Table 2, Fig. 3). *Apcs* and *Cav1* expression was downregulated by MCS in Ts mice, and *Aplp1* upregulated (Table 2, Fig. 3). Additionally, MCS significantly increased genes involved in APP processing and transport (presenilin 2, *Psen2*; low density lipoprotein receptor-related protein 1, *Lrp1*; membrane metallo endopeptidase, *Mme*), intracellular signaling (cyclin-dependent kinase 5 regulatory subunit 1 p35, *Cdk5r1*; APP binding family A member 1, *Apba1*), AD plaque constituent (beta-2 microglobulin, *B2m*; synuclein beta, *Sncb*), as well as perlecan/heparan sulfate proteoglycan 2 (*Hspg2*), bromodomain PHD finger transcription factor (*Bptf*), and versican (*Vcan*), but significantly decreased NEDD8 activating enzyme E1 subunit 1 (*Nae1*) in Ts mice (Table 2). Expression of *Cav1*, *Aplp2*, and microtubule-associated protein tau (*Mapt2*) was upregulated and *Ncstn* downregulated by MCS in 2N mice (Table 2).

Alterations in cholinergic and galaninergic transcripts

Nicotinic cholinergic receptor (nAChR) beta polypeptide 3 (*Chrnb3*) transcript levels were upregulated in Ts mice, and MCS normalized *Chrnb3* expression to 2N levels (Fig. 4). No statistically significant genotype changes were seen in the family of muscarinic receptors (mAChRs) or cholinergic synthesizing and transport genes between genotypes in the normal diet condition. MCS upregulated expression of nAChR alpha polypeptides 3 (*Chrna3*) and 7 (*Chrna7*), beta polypeptides 1 (*Chrnb1*) and 2 (*Chrnb2*), and mAChR 1 (*Chrm1*) and 2 (*Chrm2*) in Ts mice (Fig. 4). *Chrnb3* was also significantly upregulated by MCS in 2N mice (Fig. 4).

Galanin (*Gal*), a gene encoding the neuropeptide Gal that co-localizes with BFCNs in rodents (Perez, Wynick, Steiner, & Mufson, 2001), was significantly upregulated in Ts mice (Fig. 4). Gal receptor (GalR) transcript levels were similar between genotypes under normal diet conditions. MCS downregulated *Galr2* expression in Ts mice, and upregulated expression of *Gal* in 2N mice.

Immediate-early gene transcripts

Immediate early genes FBJ osteosarcoma oncogene B (*Fosb*) and activity regulated cytoskeletal-associated protein (*Arc*) were significantly upregulated in Ts mice. MCS

significantly decreased expression of *Fosb* and *Arc* as well as cAMP responsive element binding protein 1 (*Creb1*), although levels of *Fosb* remained 2.5-fold higher that 2N mice ($p < 0.07$) and *Arc* 4.2-fold higher (Table 3, Fig. 5). *Arc* was significantly upregulated by MCS in 2N mice (Table 3, Fig. 5).

Intracellular signaling related transcripts

Of the 55 intracellular signaling related transcripts examined, six were significantly increased in Ts compared with 2N mice, including *Aplp1* and *Aplp2* genes associated with AD (Table 2, Fig. 3), regulator of G-protein signaling 4 (*Rgs4*), 5 (*Rgs5*), and 10 (*Rgs10*), and gas neurotransmitter nitric oxide synthase 2 inducible (*Nos2*) (Table 3), whereas three intracellular signaling genes were significantly decreased inositol 1,4,5-trisphosphate 3-kinase A (*Itpka*); and G-protein related MTOR associated protein LST8 homolog, *Mlst8*; and G-protein gamma 3, *Gng3*) (Table 3). No genes associated with adenylyl cyclase (AC) signaling on the custom-designed array were significantly changed across genotype under normal diet conditions. MCS normalized expression levels of *Rgs10* (5.40-fold decrease) and *Itpka*, *Mlst8*, and *Gng3* (average 5.90-fold increase) in Ts mice (Table 3). There were twelve genes with equivalent expression levels between 2N and Ts mice but altered by MCS in Ts mice, including the tuberous sclerosis 2 (*Tsc2*) gene implicated in ID (Table 3), and the AD associated gene *Cdk5r1* (Table 2).

Cell death-related transcripts

Of the 27 gene probes on the custom-designed microarray related to cell death signaling, expression levels of B cell leukemia/lymphoma 2 (*Bcl2*), cathepsin C (*Ctsc*), and tumor necrosis factor (TNF) receptor superfamily member 1a (TNFRSF1A)-associated via death domain (*Tradd*) were upregulated in Ts mice, and cyclin G1 (*Ccng1*) was downregulated (Table 4, Fig. 5B). MCS partially reversed these differences in expression for *Bcl2* and *Ccng1* in Ts mice, although levels were still significantly different from 2N mice (Table 4, Fig. 5B). MCS significantly increased expression of five genes in Ts65Dn mice: Fas ligand/TNF superfamily member 6 (*Fasl*), caspase 2 (*Casp2*), *Tnfrsf1a*, BCL2-associated X protein (*Bax*), apoptosis-inducing factor mitochondrion-associated 1 (*Aifm1*) (Table 4, Fig. 5B). MCS significantly increased expression of *Bcl2* in 2N mice (Table 4, Fig. 5B). Aside from *Bcl2* in Ts mice, no genes associated with cell death were decreased with MCS in either genotype.

Cytoskeletal and presynaptic transcripts

There were 28 probes related to neuronal cytoskeleton and associated structural proteins. Upregulation of tubulin beta 4B class IVB (*Tubb4b*) and gamma-aminobutyric acid (GABA) receptor associated protein (*Gabarap*) was found in Ts compared to 2N mice (Table 5; Fig. 5C). Cytoskeletal elements dynein cytoplasmic 1 heavy chain 1 (*Dync1h1*) and synaptopodin (*Synpo*) were significantly decreased in Ts compared to 2N mice (Table 5; Fig. 5C). Expression levels of *Tubb4b*, *Gabarap*, and *Dync1h1* were normalized by MCS. MCS treatment produced an upregulation of caveolin 2 (*Cav2*), caveolin 3 (*Cav3*), drebrin 1 (*Dbn1*), synuclein gamma (*Sncg*), and doublecortin (*Dcx*) expression in Ts mice (Fig. 5C) but significantly upregulated *Tubb4b* and *Gabarap*, and downregulated *Synpo* in 2N mice (Table 5; Fig. 5C).

Of the genes related to presynaptic function, upregulation of synaptogyrin 1 (*Syngr1*) and downregulation of synuclein, alpha (*Snca*) was found in Ts mice (Table 5). MCS significantly decreased expression of *Syngr1* and bassoon (*Bsn*) in Ts mice; while in 2N mice, MCS upregulated synaptosomal-associated protein 29 (*Snap29*) and downregulated *Snca* (Table 5).

Autophagosome and protein degradation transcripts

Expression of multiple autophagosome-associated genes was significantly increased in Ts compared with 2N mice including autophagy related beclin 1 (*Becn1*) and those encoding for proteins involved in the ubiquitin degradation process: cyclin F (*Ccnf*), heat shock protein 1A (*Hspa1a*), and ubiquitin specific peptidase 8 (*Usp8*). Additionally, we found an increase in exosomal member of RAS oncogene family 27a (*Rab27a*) and a decrease in endosomal *Rab11b* in Ts compared to 2N mice (Fig. 6A). In Ts mice, MCS decreased expression of *Becn1*, *Ccnf*, *Hspa1a*, *Usp8*, and *Rab27a* (Fig. 6A), and in 2N mice of *Rab11b* (Fig. 6A). In addition MCS increased expression of lysosomal and autophagic cathepsin B (*Ctsb*), D (*Ctsd*), E (*Ctse*), and G (*Ctsg*), autophagy related 3 (*Atg3*) and 5 (*Atg5*), autophagy related cysteine peptidase 4A (*Atg4a*) and 4D (*Atg4d*), catalase (*Cat*), protein phosphatase 2 regulatory subunit B' gamma (*Ppp2r5c*), insulin-like growth factor 2 receptor (*Igf2r*), ubiquitin-conjugated enzyme E2E 1 (*Ube2e1*), and *Rab9*, as well as endosomal mannose-6-phosphate receptor (*M6pr*) and sortilin 1 (*Sort1*) (Figure 6B).

Transcript expression validation

Validation of select transcripts in MS/VDB BFCNs was performed by NanoString nCounter through hippocampal CA1 subregional analysis (a target for MS/VDB cholinergic projections), not single population analysis, as part of our ongoing studies of the septohippocampal system in Ts and 2N offspring following MCS. NanoString nCounter hippocampal CA1 subregional analysis revealed expression level changes in *Synpo* (downregulation) and *Psen2* (upregulation) concurrent with the findings reported using custom-designed microarrays. Specifically, downregulation of *Synpo* in Ts compared with 2N mice found via microarray paralleled significant downregulation of CA1 *Synpo* transcript levels in Ts compared with 2N mice via NanoString nCounter ($p < 0.007$). Moreover, downregulation of *Synpo* by MCS in Ts BFCNs mirrored significant downregulation of *Synpo* in hippocampal CA1 subregional analysis of Ts+ mice ($p < 0.01$). Further, upregulation of *Psen2* by MCS in BFCNs was also validated by NanoString nCounter of *Psen2* in hippocampal CA1 subregional analysis of Ts+ compared with Ts mice ($p < 0.02$). Frozen tissue from the MS/VDB region was not available for transcript expression validation.

Discussion

In DS there is a striking loss of cholinergic neurons located within the basal forebrain (Coyle, Oster-Granite, & Gearhart, 1986; Fodale, Mafrica, Caminiti, & Grasso, 2006; Schliebs & Arendt, 2011), an area that provides the major cholinergic input to the hippocampal component of the medial temporal lobe memory circuit (Berger-Sweeney, 2003; Hampel et al., 2018; Hasselmo & Sarter, 2011; Mesulam et al., 1983; Whitehouse et

al., 1983). Recently, studies have demonstrated that dietary MCS during pregnancy and lactation attenuates cognitive dysfunction and BFCN degeneration in adult Ts65Dn progeny (Ash et al., 2014; Kelley et al., 2016; Kelley et al., 2014a; Moon et al., 2010; Powers et al., 2017; Strupp et al., 2016). Despite the importance of the cholinergic basal forebrain projection system in cognitive function, little is known about the molecular pathobiology of BFCNs in DS (Alldred et al., 2018; Alldred et al., 2015a, 2015b; Chrast et al., 2000; Perez et al., 2019). Here, we present single population neuronal expression data showing alterations to the transcriptional signature of MS/VDB cholinergic neurons obtained from offspring of Ts65Dn dams treated with MCS or a choline-controlled diet during gestation and nursing. Overall, we found genotype-dependent alterations in select transcripts from gene ontology categories related to AD, G-protein intracellular signaling, immediate early gene responses, cell death, cytoskeletal structure and transport, autophagy, protein degradation, presynaptic function, neurotransmission, and neuromodulation. Interestingly, MCS corrected aberrant expression of various genes in cytoskeletal, G-protein intracellular signaling, immediate early, and cell death functional pathways.

Consistent with prior reports in humans with DS and in Ts mice, we did not find a gene dosage effect for the triplicated segment in Ts mice (Choi et al., 2009; Gardiner, 2004; Holtzman et al., 1995; Pritchard & Kola, 1999). For example, expression levels for the *App* gene were comparable between 2N and Ts mice; and MCS had a differential effect, increasing *App* expression in Ts+, while levels were unchanged in 2N+ mice. Although elevated levels of the protein product APP have been reported in Ts mice, data do not suggest an increase in Aβ processing enzymes or in the deposition of Aβ moieties; moreover, *App* mRNA has a shorter half-life in Ts than 2N mice (Choi et al., 2009; Holtzman et al., 1996; Hunter et al., 2003; Salehi et al., 2006). In an APP/PS1 AD mouse model, MCS resulted in decreased levels of soluble Aβ40 and Aβ42 peptides and reduced size of amyloid-like plaques in the hippocampus (Mellott et al., 2017), suggesting there may be further downstream effects in addition to the mRNA changes we found.

We previously reported ChAT activity increases in the hippocampus at 15–19 months of age in Ts and Ts+ compared with treatment-matched 2N mice (Kelley et al., 2016), corresponding with reports from independent labs of increases in ChAT enzyme activity at 10–12 months of age in the hippocampus, olfactory bulb, and isocortex of Ts compared with 2N mice (Contestabile et al., 2006; Seo & Isacson, 2005). In the present study, we did not find genotype-specific alterations in cholinergic synthesizing (choline acetyltransferase, *Chat*), transport (solute carrier family 18 vesicular monoamine member 3, *Slc18a3*), or degradation (acetylcholinesterase, *Ache*; butyrylcholinesterase, *Bche*) transcripts within BFCNs in Ts compared to 2N mice at 4–8 months of age, suggesting enzyme activity changes are either age dependent or occur via a mechanism other than direct mRNA regulation in MS/VDB cholinergic neurons. In Ts offspring exposed to MCS there was no change in *Chat*, *Slc18a3*, or *Ache*, but there was an upregulation in several nAChR and mAChR genes, suggesting alterations in local network signaling may occur at the transcription level, while ACh metabolic factors remain unaltered. Prior work in disomic rats has shown that MCS results in a functional profile of decreased ACh turnover (marked by decreased protein activity levels of ChAT, AChE, and high-affinity choline uptake) along with increased evoked ACh release, in the hippocampus during the first two months of life

(Blusztajn et al., 1998; Cermak et al., 1999; Cermak, Holler, Jackson, & Blusztajn, 1998; Meck, Williams, Cermak, & Blusztajn, 2007). Whether the changes observed in disomic rats with MCS persist throughout adult life and whether they are discoverable in BFCNs is unknown (Cermak et al., 1999). We did not find transcript changes in the MS/VDB of 2N mice that mirror activity alterations in the hippocampus of MCS exposed rats, suggesting that if a similar downregulation occurs in the current model, it is not at the transcription level in these neurons. There are conflicting reports on whether the forebrain cholinergic system of individuals with DS is altered from birth (Becker, et al., 1991; Kish, et al., 1989; Casanova, et al., 1985; McGeer, et al., 1985).

Alterations in intracellular signaling transcripts within Ts BFCNs compared with 2N mice may provide insight into the failure of the septohippocampal system to respond appropriately to stimuli in trisomic mice. A prior study found no difference in hippocampal ACh baseline release rates in Ts compared to 2N mice (4 months of age), but a significantly greater release rate in 2N mice during the performance of a hippocampal dependent memory-attention task that was not seen in Ts mice (Chang & Gold, 2008). In the present study, expression of two immediate early genes, *Fosb* and *Arc*, were significantly elevated in Ts mice, and significantly decreased with MCS. The genotype-dependent elevation in immediate early genes is reminiscent of previously reported increases in protein levels for proto-oncogene c-Fos (FOS), phosphorylated FOS (pFOS), and ARC in the hippocampus of 7-month-old Tc1 mice, a murine model of DS with a transchromosomic copy of HSA21 (Ahmed et al., 2013). The fact that we did not find Ts-specific elevations in *Fos*, fos-like antigen 2 (*Fosl2*), or other immediate early genes suggests a specific dysregulation of *Arc* and *Fosb* transcription within BFCNs in the Ts65Dn mouse. Of note, the protein encoded by *Arc* interacts with mRNA or the lipid bilayer and is involved in long-term depression at the synapse whereas the other immediate early genes examined function through polymerase and transcription factor binding, related to nuclear regulatory roles prior to mRNA turnover (Barylko et al., 2018; Dynes & Steward, 2012; Minatohara, Akiyoshi, & Okuno, 2015).

The synaptic-related markers, *Snca* and *Syngr1*, were significantly altered in Ts mice and normalized with MCS. Functionally, *Snca* encodes alpha synuclein, a protein that is involved in presynaptic signaling and vesicle trafficking, and an effector in the pathogenesis of an autosomal dominant form of Parkinson disease (PD), as well as a component of Lewy bodies in sporadic PD and amyloid beta plaques in AD (Kessler et al., 2018; Mukaetova-Ladinska et al., 2000; Xu & Pu, 2016). *Syngr1* encodes synaptogyrin 1, a protein involved in vesicle trafficking and synaptic plasticity (Belfort & Kandror, 2003; Janz et al., 1999). The ability of MCS to normalize expression of these synaptic genes may partially underlie the prolonged benefits of MCS on septohippocampal-dependent memory tests seen at 6–12 months in Ts mice (Ash et al., 2014; Kelley et al., 2016; Strupp et al., 2016). Of note, alpha synuclein and synaptogyrin are not involved in vesicle docking or release and genotype dependent differences represent a problem with vesicle maintenance and synaptic plasticity rather than neurotransmitter exocytosis (Ben Gedalya et al., 2009; Janz et al., 1999; Vargas et al., 2017).

Analysis of cell stress and apoptosis related transcripts revealed an increase in the expression of *Bcl2*, *Ctsc*, and *Tradd* in Ts compared with 2N mice. *Bcl2* encodes an outer mitochondrial

membrane protein that can regulate apoptosis by controlling permeability, while *Ctsc* encodes the protein cathepsin C, which is involved in peptidase regulation and proteolysis associated with the apoptotic process. B*y* contrast, *Tradd* is involved in extrinsic apoptotic signaling as a cytoplasmic effector for death domain-containing receptors. Functionally, an upregulation of *Tradd*, suggests that the TNF or Fas signaling pathway is activated, however we did not find alterations in downstream effectors such as *Casp3*, *Casp7* or *Bad*. We also found a significant decrease between Ts and 2N mice for *Ccng1* expression, which encodes cyclin G1, a negative regulator of apoptosis. MCS normalized expression of *Bcl2* and *Ccng1* without significantly altering *Ctsc* or *Tradd* expression levels in Ts mice. Alterations in these cell survival genes suggest either an ongoing neurodegenerative stress response or a system that remains in an immature state. MCS exposure in Ts mice increased expression of genes involved in cytochrome C mediated stress responses and apoptosis including pro-apoptotic *Bax* and *Casp2*. However, there were no alterations in downstream effectors, suggesting that while a stress response may be ongoing, apoptotic processes are quiescent in MCS exposed Ts mice.

Although prior studies in this mouse model have focused on increases in protein markers for early endosomes, such as *Rab5b*, as indicative of neuronal transport dysfunction (Cataldo et al., 2003; Delcroix et al., 2004; Ginsberg, Alldred, et al., 2010; Salehi et al., 2006), we did not find any genotype specific changes in this class of genes. In the present study, we found increased expression of genes coding for protein products involved in the ubiquitin degradation system (*Usp8*, *Ccnf*, *Hspa1a*) and autophagy (*Becn1*), and decreased expression of *Rab11b*, protein product involved in receptor recycling, in Ts compared to 2N mice. *Hspa1a* is elevated in response to oxidative stress and misfolded proteins, and protein expression is increased in the deposits of AD and other neurodegenerative diseases (Hauser et al., 2005; Leak, 2014; Leverenz, Miller, Dobie, Peskind, & Raskind, 2001). Notably we found that MCS corrected elevated levels of *Hspa1a* in Ts while not altering expression levels in 2N mice.

Interestingly, there was a significant increase in gene expression of the neuropeptide Gal in Ts mice compared with 2N mice. Gal and GalRs co-localize with BFCNs during development and into adulthood in rodents (Chan-Palay, 1988; Melander et al., 1985; Mufson, Cochran, Benzing, & Kordower, 1993; Perez et al., 2001; Vogels, Renkawek, Broere, ter Laak, & van Workum, 1989) and BFCN degeneration is associated with an increase in Gal and GalRs as well as hypertrophy of galaninergic fibers (Beal, MacGarvey, & Swartz, 1990; Chan-Palay, 1988; Counts, He, Che, Ginsberg, & Mufson, 2009; Kelley et al., 2011; Mufson, Counts, Perez, & Binder, 2005; Sendera et al., 2000). Functionally, Gal may be neuroprotective by preventing Aβ-induced cell death or by decreasing cleavage of caspase 3 and caspase 9 in AD (Ding, MacTavish, Kar, & Jhamandas, 2006; Elliott-Hunt et al., 2011; Vogels et al., 1989). Whether the increase in Gal expression is triggered by an extracellular or inter-cellular event, or is a developmentally related transcriptional bystander effect remains to be investigated. While *Gal* expression was not significantly altered with MCS in TS mice, we found the expression *Galr2* was significantly decreased with MCS in Ts mice. Prior studies have highlighted GalR2 agonists as providing the same neuroprotection as Gal (Ding et al., 2006; Sipkova et al., 2017), and based on its role in

disease pathology, this can be a general indicator of the health of the MS/VDB system (Beal et al., 1990).

In the present study, we observed comparable Cdk5 expression levels between Ts and 2N animals, but a paradoxical increase in MCS treated Ts mice. Cdk5, which encodes for a serine/threonine kinase involved in cytoskeletal dynamics, plays a role brain development (Yoo and Lubec, 2001) and in the formation of tau pathology in AD and neurodegeneration in other diseases (Bu, Li, Davies, & Vincent, 2002; Imahori & Uchida, 1997; Kimura, Ishiguro, & Hisanaga, 2014; Nguyen, Lariviere, & Julien, 2001; Patrick et al., 1999). A prior study revealed no change in hippocampal CDK5 levels at 7 months in the Tc1 mouse model of DS (Ahmed et al., 2013). On the other hand, CDK5 protein levels were increased in the hippocampus at 4 months of age, with no change in functional markers p25 and p35 in Ts mice (Cruz & Tsai, 2004; Pollonini et al., 2008; Yoo & Lubec, 2001). Interestingly, it has been reported that the amount of p25 (marker of CDK5 activity and neurotoxicity) in the frontal cortex of patients with AD or DS is lower than in controls (Yoo and Lubec, 2001). Although the sample size was small, the study raises questions as to the exact role that CDK5 and its regulatory subunits play in DS pathobiology.

Potential limitations

In the present study, we relied on mRNA extraction from fixed mouse brains to examine relative gene expression levels across groups. This type of single-time-point analysis assumes the sample is representative of a steady-state measurement. Based on prior studies, the average half-life of mRNA is estimated at 10 hours, and transcripts that have a high production and high decay rate (< 2 h half-life) may be functionally different from longer-lived transcripts but are expected to maintain a steady state expression level (Lam et al., 2001; Yang et al., 2003). With this in mind, we minimized stress by deeply anesthetizing each animal prior to entering the perfusion suite, and circadian rhythm interactions by conducting randomized perfusions across treatment groups on the same day within a 5-hour window. With regard to the latter, we did not find significant differences between groups in protein phosphatase 1 catalytic subunit genes (*Ppp1ca*, *Ppp1cb*, and *Ppp1cc*), which play a role in circadian regulation and photoperiod entrainment. However, we cannot rule out effects due to intrinsic factors such as possible polymorphisms in select genes that affect turnover rates (Puga et al., 2005), which are unexplored in this mouse model, and the custom-designed array platform is not designed to detect.

It is important to bear in mind that the Ts65Dn murine model is representative of a specific pathology found in DS and AD, notably age-related degeneration of the septohippocampal cholinergic system. This model lacks the classic neuropathological hallmarks of aging DS and AD, namely amyloid plaques and neurofibrillary tangles. Moreover, the model does not recreate the full genetic condition of human trisomy 21; several genes triplicated in humans with DS are not present in the Ts65Dn model and there are triplicated genes in Ts65Dn mice that are not trisomic in human DS (Sturgeon & Gardiner, 2011). These considerations should be kept in mind when extrapolating the present findings to other species and treatment models. Despite this caveat, the Ts65Dn mouse does provide a unique model with reproducibility for the study of the cellular and molecular pathobiology that trisomy exerts

upon the septohippocampal memory circuit. Expression profiles reported herein implicate key signaling pathways beyond canonical AD-related genes in the pathogenesis of MS/VDB neurons in DS and AD. The datasets derived using a vulnerable-cell specific population provide tangible targets related to mechanistic alterations and functional consequences of septohippocampal degeneration. These investigations begin the process of defining which transcript (and ultimately encoded proteins) changes are primary and which are secondary within BFCNs in response to MCS in Ts65Dn mice, which has direct implications for translation to human disease.

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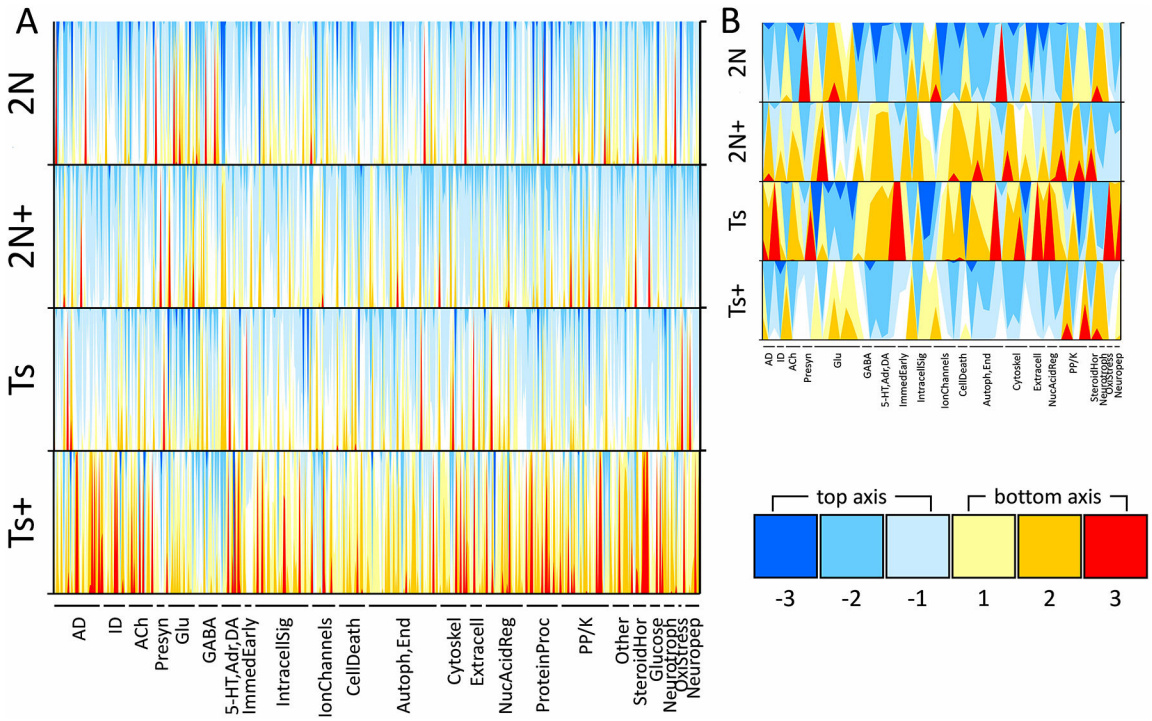

Figure1.

Horizon plots display transcription signature of MS/VDB basal forebrain cholinergic neurons from Ts65Dn (Ts) and disomic (2N) mice exposed to maternal choline supplementation (MCS, +) or normal diet from conception to weaning. **A.** Two-axis horizon plots show the relative expression level of all genes examined, using normalized row (gene) z-scores across the four groups. Negative values descend from the top abscissa (blues) and positive values ascend from the lower abscissa (reds and yellows) as shown in the legend at the lower right. Horizon plots stack data to provide an illustration for intensity of differences across genotype and treatment groups by overlaying fold-change surface plots and color-coding for overt determination of patterns. Each stacked surface plot (i.e. each color) represents a one-fold change, such that the lighter shades (-1 and 1 in the legend) are nearer to the mean expression which is set at 0, and darker shades descend/ascend accordingly (−2, −3 and 2, 3 in the legend). Use of two x-axes prevents overlap of negative and positive z-scores. Gene classes are listed below the 4 horizon plots and each spike can be thought of as a gene in that class (AD, Alzheimer's disease; ID, intellectual disability; ACh, acetylcholine; Presyn, presynaptic; Glu, glutamate; GABA, gamma aminobutyric acid; 5-HT,Adr,DA, serotoninergic, adrenergic, and dopaminergic; IntracellSig, intracellular signaling; Autoph,End, autophagy and endosomal; Cytoskel, cytoskeleton; Extracell, extracellular matrix and nontransmitter inter-cellular signaling; NucAcidReg, nucleic acid regulation; ProteinProc, protein processing; PP/K, protein phosphatase and kinase; SteroidHor, steroid hormones; Neurotroph, neurotrophins; OxiStress, oxidative stress; Neuropep, neuropeptides). **B.** The horizon plot that results when only the genes with significant genotype differences in normal diet conditions (2N × Ts) as well as significant alteration with MCS in Ts mice (Ts+ × Ts) is shown ($p < 0.01$ using mixed effects model with false discovery rate based on null distribution; 10 mice per group). As opposed to the overall

expression differences shown in **A**, the plots in **B** shows a clear expression pattern of elevated levels in Ts and 2N+ mice and lower levels in 2N and Ts+ mice, providing an overview of the genotype and treatment interaction effect in this murine model with MCS.

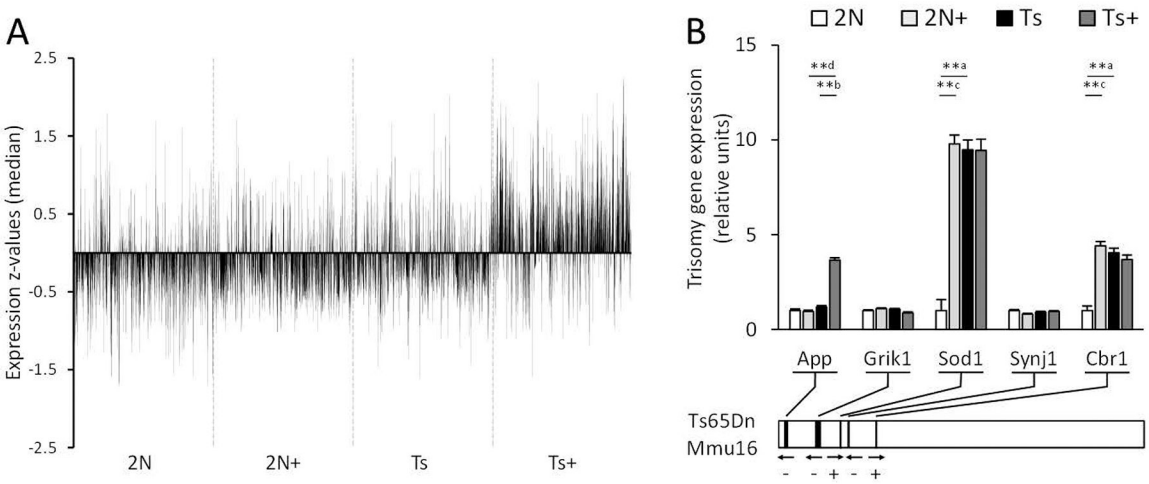

Figure 2.

Total expression and trisomy related gene expression levels of genes on a custom-designed microarray were measured in isolated basal forebrain cholinergic neurons from the MS/VDB. **A.** Vertical spike plot shows median z-scores per probe from a membrane microarray analysis on cholinergic neurons in the MS/VDB of Ts65Dn mice (Ts) and disomic littermates (2N). From conception to weaning, dams and pups were exposed to either normal diet or maternal choline supplementation (+). There are clear treatment and genotype effects when transcription comparison results are examined as a whole, including an overall increase in expression of Ts+ mice genes compared to the other groups. **B.** Bar graph shows expression levels of five genes triplicated in the Ts65Dn mouse model. The chromosome segment underneath diagrams to-scale locations of the genes on the chromosome. ** $p < 0.005$ using a mixed effects model with false discovery rate based on null distribution, n = 10 mice per group; a, Ts × 2N; b, Ts+ × Ts; c, 2N+ × 2N; d, Ts+ × 2N+

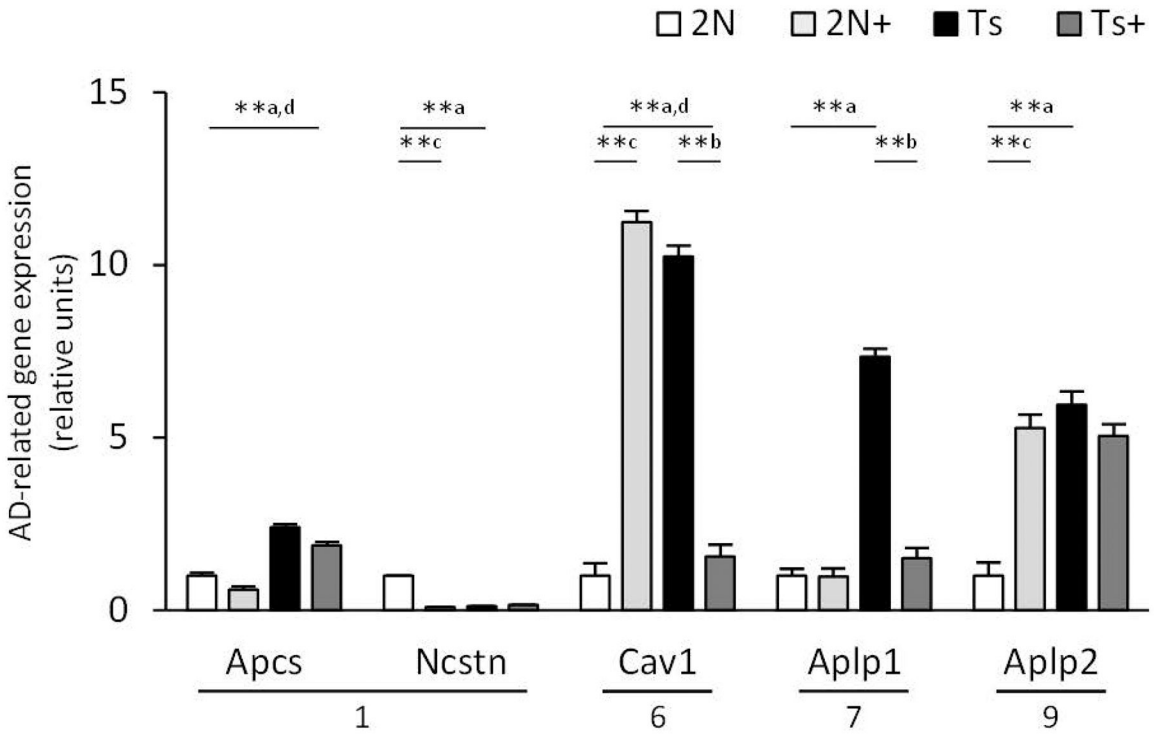

Figure 3.

Bar graph shows expression levels of Alzheimer disease (AD) related genes. Ts65Dn (Ts) and disomic (2N) mice were exposed to a maternal choline supplementation (+) or normal diet from conception to weaning and gene expression levels were measured in basal forebrain cholinergic neurons at 4.4–7.5 mos. Multiple AD-associated genes were upregulated in Ts compared with 2N mice including *Cav1* and *Aplp1*, which were downregulated in Ts+ compared with Ts mice. * $p < 0.01$, ** $p < 0.005$, mixed effects model with false discovery rate based on null distribution, n = 10 mice per group; a, Ts × 2N; b, Ts + × Ts; c, 2N+ × 2N; d, Ts+ × 2N+; chromosomes are shown under genes

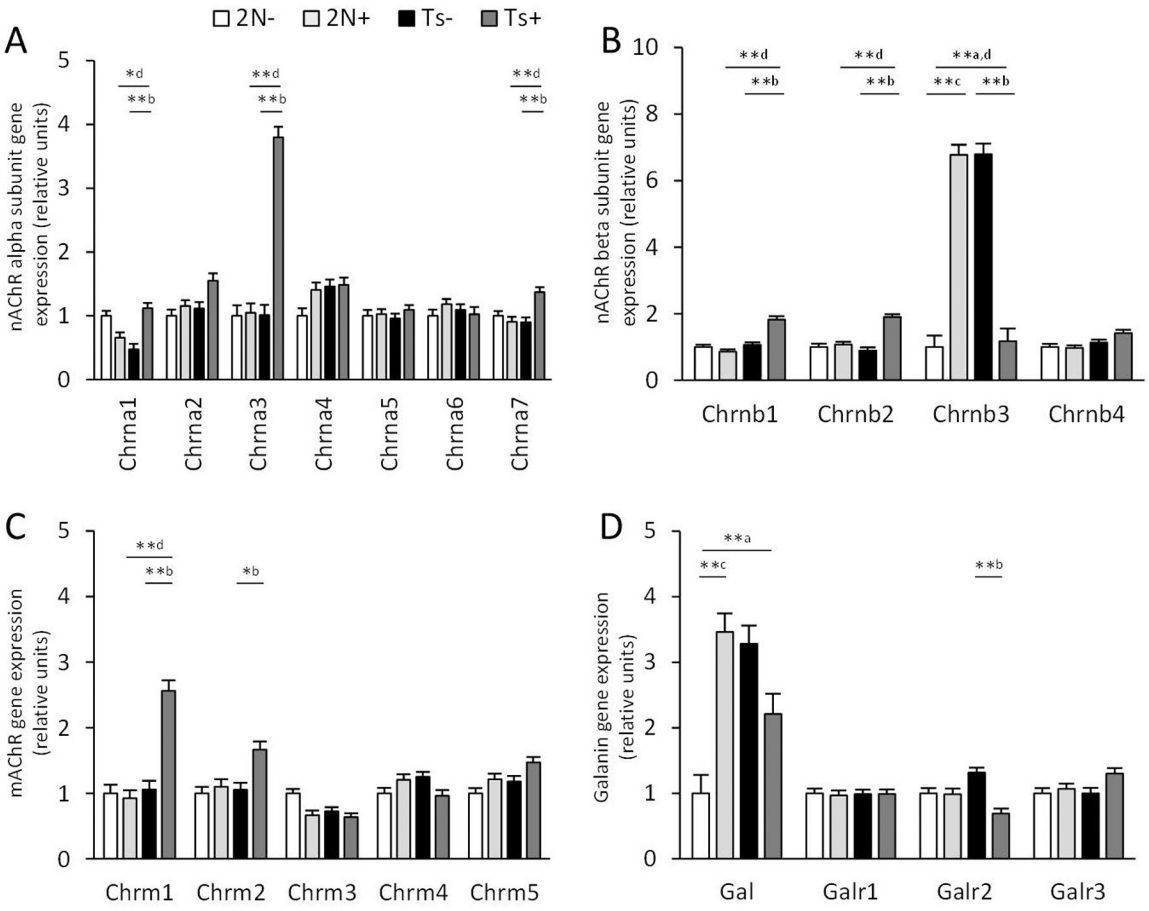

Figure 4.

Bar graph shows expression levels of cholinergic receptor (AChR) and galanin (Gal) related genes. Ts65Dn (Ts) and disomic (2N) mice were exposed to a maternal choline supplementation (+) or normal diet from conception to weaning and gene expression levels were measured in basal forebrain cholinergic neurons at 4.4–7.5 mos. **A-B.** Multiple nicotinic AChR (nAChR) alpha (*Chrna*, **A**) and beta (*Chrnb*, **B**) subunits were upregulated in Ts+ compared to Ts mice, but only *Chrnb3* was elevated in 2N+ compared with 2N mice. **C.** Muscarinic AChR (mAChR) genes (*Chrm*) were stably expressed in 2N+ and 2N mice, while *Chrm1* and *Chrm2* were significantly elevated in Ts+ compared to Ts mice. **D.** Transcripts for *Gal* were elevated in Ts compared with 2N mice but not Gal receptor genes (*Galr*). * $p < 0.01$, ** $p < 0.005$, mixed effects model with false discovery rate based on null distribution, n = 10 mice per group; a, Ts × 2N; b, Ts+ × Ts; c, 2N+ × 2N; d, Ts+ × 2N+

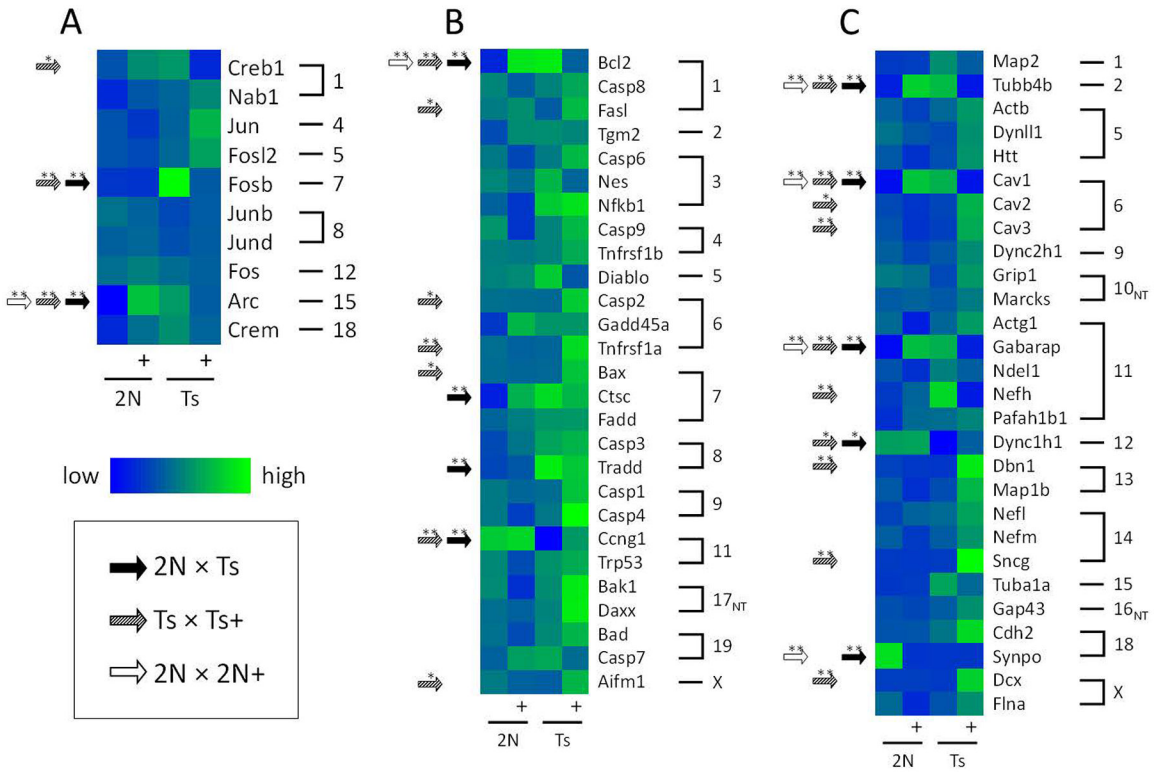

Figure 5.

Heatmaps show gene expression levels in basal forebrain cholinergic neurons for select gene classes between disomic (2N) and Ts65Dn (Ts) mice exposed to normal diet or maternal choline supplementation (+). **A.** Heatmap of immediate early gene expression levels shows significant increases in *Arc* and *Fosb* expression levels between Ts and 2N mice (black arrows) and partial normalization with MCS (hatched arrows). Lowest expression levels are in blue (*Arc*, 2N), and highest in green (*Fosb*, Ts+). **B.** Significant differences in expression levels were seen in multiple cell death associated genes between Ts and 2N mice. Partial normalization occurred with MCS for *Bcl2* and *Ccng* in Ts mice, and MCS increased expression of *Bcl2* in 2N mice (white arrows). Lowest expression levels are in blue (*Ccng1*, Ts), and highest in green (*Casp4*, Ts+). **C** Expression levels of cytoskeleton proteins associated with intraneuronal structure and transport show significant differences both structural, *Tubb4b*, and transport, *Dync1h1*, related genes in Ts compared to 2N mice. MCS resulted in normalizing decreases of Tubb4b, Cav1, and Gabarap expression levels in Ts mice, and increased expression in *Cav2*, *Cav3*, *Nefh*, *Dbn1*, *Sncg*, and *Dcx* in Ts+ compared to Ts mice. Lowest expression levels are in blue (*Dync1h1*, Ts), and highest in green (*Sncg*, Ts+). The grouping outline is based on mouse chromosome numbers, listed on the right. *, $p < 0.01$; **, $p < 0.005$, mixed effects model with false discovery rate based on null distribution

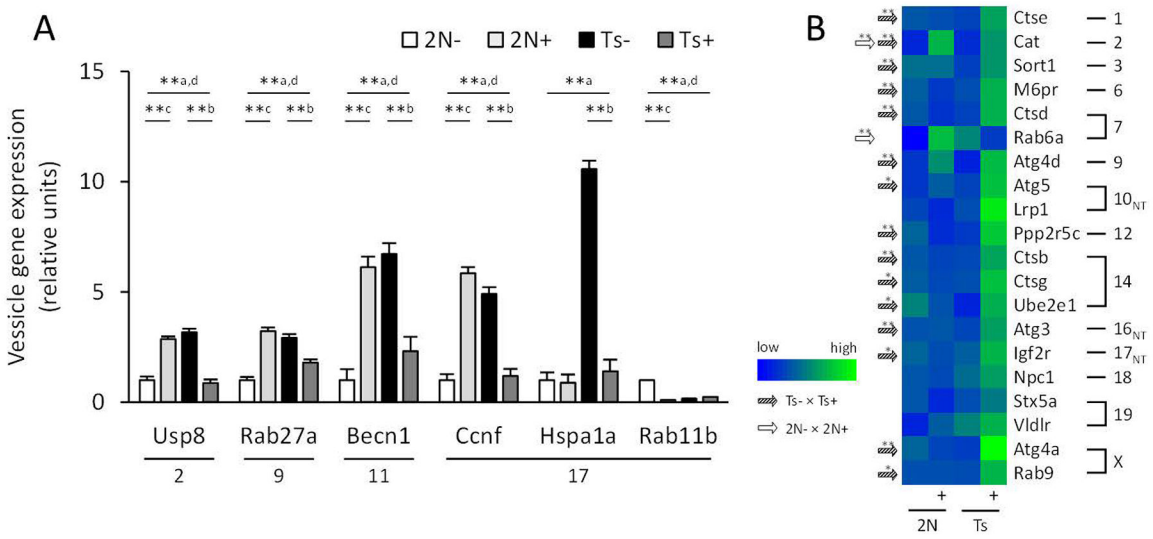

Figure 6.

Bar graph and heatmap show significant changes in gene expression levels in basal forebrain cholinergic neurons for vesicular endosomal, exosomal, and autophagy-related genes between disomic (2N) and Ts65Dn (Ts) mice exposed to normal diet or maternal choline supplementation (+). **A.** Bar graph shows increased expression of five genes related to endosomes and autophagy in Ts mice compared to 2N mice and normalization with MCS. ** $p < 0.005$ using a mixed effects model with false discovery rate based on null distribution, n = 10 mice per group; a, Ts × 2N; b, Ts+ × Ts; c, 2N+ × 2N; d, Ts+ × 2N+ **B.** Heatmap of autophagy, endosome, and exosome gene expression levels shows significant changes with MCS in Ts (hatched arrows) and 2N (white arrows) mice. Lowest expression levels are in blue (*Rab6a*, 2N), and highest in green (*Atg4a*, Ts+).

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Table 1.

Subject table

	n[†]	Age range (mean)	Sex (F:M)[‡]
2N[§]	10	4.5–7.5 (5.9) mos	8:2
2N[+]	10	4.5–7.5 (5.9) mos	7:3
Ts	10	4.5–7.1 (6.1) mos	9:1
Ts[+]	10	4.4–7.5 (6.0) mos	9:1

[†]Group sizes;

[‡]F, female; M, male;

[§]2N, disomic mice; Ts, Ts65Dn mice;

[+], maternal choline supplementation;

Table 2.

Comparison of Alzheimer's disease related gene expression levels between genotypes and treatments

Gene	Mmu†	2N+ fold of 2N‡	Ts fold of 2N	Ts+ fold of 2N	Ts+ fold of Ts
Cav1	6	11.24**	10.25**	1.56	0.15**
Aplp1	7	0.97	7.34**	1.50	0.20**
Aplp2	9	5.28**	5.95**	5.05**	0.85
Apcs	1	0.60	2.40**	1.87**	0.78
Psen2	1	1.87	1.68	2.40**	1.43*
Nae1	8	1.19	1.61	0.38	0.24**
Mapt2	11	5.93**	1.36	1.90	1.39
B2m	2	1.50	1.35	2.14**	1.59**
Sncb	13	1.17	1.19	2.80**	2.36**
App&	16	0.95	1.17	3.66**	3.12**
Apba1	19	0.98	1.06	2.37**	2.23**
Vcan	13	0.98	1.01	1.63*	1.62**
Cdk5r1	11	0.98	1.00	1.49*	1.49*
Mapt4	11	1.04	0.99	1.72**	1.74**
Mapt2n6p	11	0.89	0.97	4.97**	5.13**
Mapt1n6p	11	0.82	0.96	1.38	1.44*
Mme	3	0.92	0.95	1.46	1.54**
Lrp1	10_{NT}	0.81	0.93	1.71**	1.84**
Hspg2	4	0.90	0.82	1.36	1.66**
Bptf	11	0.63	0.74	2.29**	3.10**
Ncstn	1	0.09**	0.12**	0.15**	1.33

† *Mus musculus* chromosome using build Grmc38, NT denotes not triplicated;

‡ 2N, disomic mice, Ts, Ts65Dn,

+ maternal choline supplementation;

** , $p < 0.005$;

* , $p < 0.01$;

& gene is triplicated in Ts65Dn mice

Table 3.

Comparison of intracellular signaling related gene expression levels between genotypes and treatments

Gene	Mmu[†]	2N[+] fold of 2N[‡]	Ts fold of 2N	Ts[+] fold of 2N	Ts[+] fold of Ts
Immediate early genes					
Fosb	7	0.99	10.46**	2.52	0.24**
Arc	15	8.26**	6.50**	4.15**	0.64**
Creb1	1	1.17	1.12	0.84	0.75*
Second messenger and receptor cascades					
Rgs4	1	9.19**	8.62**	8.04**	0.93
Rgs5	1	9.40**	8.36**	7.29**	0.87
Rgs10	7	7.85**	7.62**	1.19	0.16**
Nos2	11	2.52**	2.65**	2.42**	0.91
Gnas	2	1.06	1.16	8.01**	6.94**
Rgs2	1	0.99	1.09	3.27**	3.00**
Gna11	10_{NT}	0.90	1.06	1.64*	1.54**
Nrg2	18	0.92	0.95	1.56**	1.65**
Tsc2[#]	17_{NT}	0.92	0.94	1.89*	2.01**
Adcy1	11	0.92	0.92	1.38	1.50**
Adcy5	16_{NT}	0.87	0.89	1.38	1.54**
Adcy4	14	0.92	0.88	1.31	1.49*
Adcy8	15	0.85	0.86	1.94**	2.25**
Itpr1	5	0.79	0.55	1.05	1.90*
Mlst8	17_{NT}	0.81	0.19**	0.82	4.34**
Gng3	19	0.85	0.18**	1.09	6.04**
Itpka	2	0.09**	0.11**	1.15	10.32**

[†] *Mus musculus* chromosome using build Grmc38, NT denotes not triplicated;

[‡] 2N, disomic mice, Ts, Ts65Dn,

[+], maternal choline supplementation;

**, $p < 0.005$;

*, $p < 0.01$;

[#] gene is implicated in intellectual disability

Table 4.

Comparison of cell death related gene expression levels between genotypes and treatments

Gene	Mmu[†]	2N[+] fold of 2N[‡]	Ts fold of 2N	Ts[+] fold of 2N	Ts[+] fold of Ts
Tradd	8	1.17	2.96 **	2.30 **	0.78
Bcl2	1	2.42 **	2.57 **	1.58	0.62 **
Ctsc	7	1.80	2.11 **	1.91 **	0.91
Bax	7	1.05	0.96	1.43	1.48 *
Fasl	1	1.02	0.96	1.45	1.52 *
Casp2	6	1.03	0.90	1.36	1.50 *
Tnfrsf1a	6	0.95	0.86	1.39	1.62 **
Aifm1	X	0.75	0.74	1.21	1.64 *
Ccng1	11	1.01	0.22 **	0.71 **	3.25 **

[†] *Mus musculus* chromosome using build Grmc38, NT denotes not triplicated;

[‡] 2N, disomic mice, Ts, Ts65Dn,

+, maternal choline supplementation;

**, $p < 0.005$;

*, $p < 0.01$

Table 5.

Comparison of cytoskeletal and presynaptic related gene expression levels between genotypes and treatments

Gene	Mmu†	2N+ fold of 2N‡	Ts fold of 2N	Ts+ fold of 2N	Ts+ fold of Ts
Cytoskeletal					
Gabarap	11	8.87**	8.16**	2.05	0.25**
Tubb4b	2	3.29**	3.01**	0.81	0.27**
Nefh	11	1.16	1.62	0.88	0.54**
Dbn1	13	1.05	1.13	2.13**	1.88**
Sncg	14	1.06	1.11	4.94**	4.46**
Dcx	X	0.97	1.02	2.22**	2.17**
Cav2	6	0.93	0.92	1.37	1.48*
Cav3	6	0.87	0.84	1.46**	1.74**
Dync1h1	12	0.86	0.37*	0.77	2.08*
Synpo	18	0.05**	0.06**	0.07**	1.06
Presynaptic					
Syngr1	15	0.89	14.64**	0.99	0.07**
Snap29	8	2.21**	1.13	1.38	1.22
Bsn	9	0.91	1.00	0.65	0.66*
Snca	6	0.26**	0.33**	0.61*	1.87

† *Mus musculus* chromosome using build Grmc38, NT denotes not triplicated;

‡ 2N, disomic mice, Ts, Ts65Dn,

+, maternal choline supplementation;

**, $p < 0.005$;

*, $p < 0.01$