Maternal choline supplementation in a mouse model of Down syndrome: effects on attention and nucleus basalis/substantia innominata neuron morphology in adult offspring

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Abstract

The Ts65Dn mouse model of Down syndrome (DS) and Alzheimer's disease (AD) exhibits cognitive impairment and degeneration of basal forebrain cholinergic neurons (BFCNs). Our prior studies demonstrated that maternal choline supplementation (MCS) improves attention and spatial cognition in Ts65Dn offspring, normalizes hippocampal neurogenesis, and lessens BFCN degeneration in the medial septal nucleus (MSN). Here we determined whether (*i*) BFCN degeneration contributes to attentional dysfunction, and (*ii*) whether the attentional benefits of perinatal MCS are due to changes in BFCN morphology. Ts65Dn dams were fed either a choline-supplemented or standard diet during pregnancy and lactation. Ts65Dn and disomic (2N) control offspring were tested as adults (12–17 months of age) on a series of operant attention tasks, followed by morphometric assessment of BFCNs. Ts65Dn mice demonstrated impaired learning and attention relative to 2N mice, and MCS significantly improved these functions in both genotypes. We also found, for the first time, that the number of BFCNs in the nucleus basalis of Meynert/substantia innominata (NBM/SI) was significantly *increased* in Ts65Dn mice relative to controls. In contrast, the number of BFCNs in the MSN was significantly *decreased*. Another novel finding was that the volume of BFCNs in both basal forebrain regions was significantly

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larger in Ts65Dn mice. MCS did not normalize any of these morphological abnormalities in the NBM/SI or MSN. Finally, correlational analysis revealed that attentional performance was inversely associated with BFCN volume, and positively associated with BFCN density. These results support the lifelong attentional benefits of MCS for Ts65Dn and 2N offspring and have profound implications for translation to human DS and pathology attenuation in AD.

Children with Down syndrome (DS) exhibit a range of intellectual disabilities, most prominently deficits in explicit memory, executive functions and sustained attention (Brown et al., 2003; Rowe et al., 2006). Individuals with DS nearly universally develop neuropathology associated with Alzheimer's disease (AD) by the third to fourth decade of life, including degeneration of cholinergic basal forebrain neurons (Mufson et al., 2003; Sendera et al., 2000; Whitehouse et al., 1982). No treatments currently exist which prevent, reverse, or slow these sequelae associated with DS.

An established murine model of DS, the Ts65Dn mouse (Davisson et al., 1990), provides a tool to elucidate the pathogenic processes in this disorder and test potential therapies. Ts65Dn mice have triplicated segments of mouse chromosome 16 (MMU16) and mouse chromosome 17 (MMU17), which are orthologous to human chromosome 21 (HSA21). The distal end of MMU16 is translocated to the centromeric end of MMU17, creating a small translocation chromosome (Davisson et al., 1993; Holtzman et al., 1996; Reeves et al., 1995). The segmental loci exhibits ~55% conservation of known protein coding genes between MMU16 and HSA21 (Davisson et al., 1993; Gardiner, 2003; Reeves et al., 1995; Sturgeon and Gardiner, 2011). Importantly, these mice survive into adulthood and exhibit a phenotype similar to that of humans with DS (Alldred et al., 2015a, 2015b; Galdzicki and Siarey, 2003).

Ts65Dn mice show impairments in explicit memory and spatial mapping (Granholm et al., 2000; Hyde and Crnic, 2001; Hyde et al., 2001), functions modulated by basal forebrain cholinergic neuron (BFCN) projections from the medial septal nucleus (MSN) to the hippocampus (Sofroniew et al., 1990; Swanson and Cowan, 1979). MSN neurons are intact in young Ts65Dn mice, but exhibit progressive degeneration beginning at approximately 6 months of age (Cooper et al., 2001; Granholm et al., 2000; Hamlett, et al., 2016; Holtzman et al., 1996), which coincides with a decline in memory function. Ts65Dn mice also exhibit impaired vigilance or sustained attention (Driscoll et al., 2004; Moon et al., 2010; Powers et al., 2016), a functional domain modulated by BFCN projections from the nucleus basalis of Meynert/substantia innominata (NBM/SI) to the neocortex (Chudasama et al., 2004; Mesulam et al., 1983; Rye et al., 1984). In contrast to MSN neurons, little is known about the morphology of NBM/SI cholinergic neurons in Ts65Dn mice, either early in life, or during the aging process.

Our group has been investigating maternal choline supplementation (MCS) as a potential prenatal therapeutic strategy for DS (Strupp et al., 2016). Decades of research has revealed that in normal rodents supplementing the maternal diet with additional choline during gestation and/or lactation produces lifelong improvements in spatial cognition and attention of the offspring, as well as changes in MSN cholinergic neurons and electrophysiological indices of hippocampal function (Meck and Williams, 2003; Zeisel and Niculescu, 2006). Since functional deficits associated with DS correspond with endpoints affected by MCS, we previously conducted a study to test the hypothesis that MCS would improve learning and attention in Ts65Dn offspring. This study yielded evidence for substantial cognitive benefits, as well as an improved regulation of negative affect in Ts65Dn mice (Moon et al., 2010; Strupp et al., 2016).

Subsequently, we reported that MCS improved spatial cognition and partially normalized adult hippocampal neurogenesis in Ts65Dn mice (Velazquez et al., 2013). Additionally, a stereological assessment of BFCNs (immunolabeled for choline acetyltransferase [ChAT], the pan-neurotrophin receptor p75^{NTR} [p75^{NTR}], and the cognate nerve growth factor receptor TrkA) revealed that MCS prevented age-related loss of MSN BFCNs in Ts65Dn offspring (Ash et al., 2014). Notably, these effects on hippocampal neurogenesis and protection of MSN BFCNs each significantly correlated with the improved spatial cognition seen in these animals, supporting functional relationships (Ash et al., 2014; Velazquez et al., 2013).

Herein, we present new results from additional offspring from the same litters that contributed to these two previous publications (Ash et al., 2014 and Velazquez et al., 2013). These mice were used to further characterize the effects of MCS on attentional function, and assess the relationship of this cognitive benefit to NBM/SI BFCNs, the subset of BFCNs linked to the modulation of attentional function (Chudasama et al., 2004). We utilized a series of attention tasks used in our prior studies (Driscoll et al., 2004; Moon et al., 2010; Powers et al., 2016), with the addition of a novel, more challenging attention task, designed to be more sensitive for detection of potential benefits of MCS in both Ts65Dn and disomic (2N) control offspring. Following the completion of behavioral testing, brains were immunolabeled for ChAT and p75^{NTR} for an unbiased stereological assessment of BFCN morphology in the MSN and NBM/SI.

Results of the study demonstrated that supplementing the maternal diet with additional choline significantly ameliorated learning and attentional dysfunction of Ts65Dn offspring, and also improved attentional function of the 2N offspring. Furthermore, the study revealed novel findings regarding BFCN morphology in Ts65Dn mice. Specifically, we demonstrated for the first time that the number and volume of cholinergic neurons in the NBM/SI was significantly *increased* in Ts65Dn mice compared to controls, in contrast to the *decreased* number and density of cholinergic neurons in the MSN. Unfortunately, these abnormalities were not normalized by MCS. Finally, correlational analyses provided insight into the neural basis of performance in these attention tasks, and by inference, the basis of the attentional dysfunction of the Ts65Dn mice: specifically, attentional performance was inversely associated with BFCN volume, and positively associated with BFCN density.

Experimental Procedures

Subjects

Breeder pairs (female Ts65Dn and male C57Bl/6J Eicher x C3H/HeSnJ F1 mice) were purchased from Jackson Laboratories (Bar Harbor, ME) and mated at Cornell University, Ithaca, NY. Upon arrival, breeders were randomly assigned to receive one of two choline-controlled experimental diets: 1) control diet – AIN-76A purified rodent diet containing 1.1 g/kg choline chloride, or 2) choline-supplemented diet – AIN-76A purified rodent diet containing 5.0 g/kg choline chloride (Dyets Inc., Bethlehem, PA). Note that our control diet is *not* choline-deficient, but rather supplies adequate choline, as recommended by the Food and Nutrition Board of the Institute of Medicine (IOM, 1998). The choline-supplemented diet provides approximately 4.5 times the concentration of choline consumed by our control group, and is within the range of dietary variation observed in humans (Detopoulou et al., 2008).

All mice were provided *ad libitum* access to water and their assigned diets. Standard cages contained paper bedding and several objects (e.g., plastic igloo, t-tube, plastic-gel bone, and cotton square). Mice were maintained on a 12-hour light-dark cycle under temperature- and humidity-controlled conditions.

Females and males were housed individually for one week prior to pairing. Pairs remained together and produced up to 3 litters, providing subjects for this study and others (Ash et al., 2014; Kelley et al., 2014a; Velazquez et al., 2013). All subjects were selected from litters of 3–8 pups. Offspring were weaned on postnatal day 21 and provided *ad libitum* access to water and the control diet. Thus, choline-supplemented offspring were exposed to this diet only until weaning. Choline supplementation was principally through maternal exposure, although it is possible that offspring were capable of accessing the diet in the days just prior to weaning.

Ear punch tissue was sent to Jackson Laboratories (Bar Harbor, ME) for genotyping by quantitative polymerase chain reaction (qPCR) for the detection of the extra chromosomal segment and determination of Pde6B^{rd1} homozygosity. This recessive mutation leads to retinal degeneration (Bowes et al., 1993). Pde6B^{rd1} homozygous mice were excluded.

For behavioral testing, we selected 16 male mice from each of the four groups: 1) disomic offspring of dams on the control diet (*2N*), 2) Ts65Dn offspring of dams on the control diet (*Ts*), 3) disomic offspring of dams on the choline-supplemented diet (*2N+*), and 4) Ts65Dn offspring of dams on the choline-supplemented diet (*Ts+*). One month prior to testing, at approximately 11 months of age, mice were moved to a room with a 13:11-hour reversed light-dark cycle (lights off at 8:00 a.m., lights on at 9:00 p.m.), and were singly-housed to prevent fighting, which can occur when group-housed male mice of this strain are returned to the home cage following test sessions. At this time, mice were placed on a food restriction regimen to ensure motivation for food rewards during testing. Target weights were calculated at approximately 85% of their *ad libitum* weight.

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

Behavioral testing

The testing apparatus has been described in our earlier publications (Driscoll et al., 2004; Moon et al., 2010; Powers et al., 2016). Briefly, mice were tested individually in one of eight computer-controlled chambers. Chambers were manufactured in-house, with the body constructed by Ithaca Plastics Inc. (Ithaca, NY). One wall contained a retractable metal door, controlling access to a dipper (ENV0302M, MED Associates, East Fairfield, VT) that dispensed the liquid reward (Liquefied AIN-76A; Bio-Serv, Frenchtown, NJ). The opposite wall contained five nosepoke response ports, each with a green 4-mA LED embedded on the back surface.

At approximately 12 months of age, male mice began training on a series of visual discrimination and attention tasks. At this age, Ts65Dn mice have significant cognitive impairment and BFCN degeneration (Granholm et al., 2000; Holtzman et al., 1996; Hunter et al., 2004). Mice were pseudo-randomly assigned to chambers such that an equal number of mice from each group were tested in each chamber. Mice were weighed and then placed into the chambers by individuals blind to their genotype and perinatal choline treatment condition. Each mouse was weighed after each test session, returned to its home cage, and fed 30 min later (subtracting the number of calories obtained as reward during testing from the daily allowance of chow). Chambers were thoroughly cleaned following testing of each mouse, using Odormute (R.C. Steele Co., Brockport, NY). All mice were tested once per day, six days per week, and each session lasted 30 minutes or 70 trials, whichever came first. Mice progressed sequentially through the series of tasks described below. Total duration of testing was approximately 5 months.

Training

A series of four training tasks was designed to familiarize the mice with the testing chamber and the sequence of responses necessary to complete trials in the visual attention tasks (Driscoll et al., 2004). Across these four stages the mice learned that: 1) the door to the dipper alcove is raised before each trial; 2) a nosepoke into the alcove initiates a trial and triggers the door to close; and 3) following trial initiation a nosepoke into one of the five response ports triggers the door to open and the delivery of 0.01 ml of the liquid diet (i.e., reward). During the final training stage, each animal was required to respond for a fixed number of trials at each of the five response ports, to eliminate preferences or aversions to any of the ports.

Initial visual discrimination task and error types

Upon trial initiation, one of the five port LEDs was illuminated, with the location of the cue pseudo-randomized across trials such that the number of cue presentations in each port was balanced within each session. A 1-s delay separated trial initiation and cue onset, allowing mice the time to turn around and orient toward the ports before cue illumination. The cue remained illuminated for 32-s or until a response was made. A nosepoke into the illuminated

port triggered the opening of the alcove door and the presentation of the food reward. Three types of errors were distinguished: (1) *premature response*: a nosepoke into any response port prior to cue onset; (2) *inaccurate response*: a nosepoke made following cue onset but to one of the non-illuminated ports; and (3) *omission error*: failing to respond to any response port within 5-s of cue light termination. All trials in which the mouse made an initiation poke into the alcove (regardless of outcome) were defined as response trials. A failure to initiate a trial within 60-s was defined as a nontrial. An error or nontrial triggered a 5-s timeout period, signaled by the illumination of a 3-W house light on the ceiling of the chamber. A 5-s interval separated adjacent trials. Each mouse remained on this task until reaching a criterion of 80% correct responses for two out of three consecutive sessions, each consisting of at least 50 response trials. Subsequent attention tasks were fundamentally the same as this initial visual discrimination task, but with specific changes to the stimulus parameters to increase the attentional demands. The characteristics of these tasks are described below.

Attention Tasks 1 and 2

After the rules of the initial visual discrimination task had been mastered, mice were tested sequentially on two variations that were identical except that the duration of cue illumination was shortened to 2-s and 1-s, respectively. Mice were tested for 8 sessions on each task, regardless of performance.

Attention Task 3

In the next task, cue duration was 1-s, and a variable delay of 0, 2, or 4-s was imposed between trial initiation and cue illumination. These different pre-cue delay durations were presented pseudo-randomly across trials, such that each combination of delay and cued port was balanced across each session. If a response was made prior to cue onset (premature response), the trial was terminated and no cue was presented. This task tapped learning, inhibitory control, and sustained attention. Mice were tested for 18 sessions, regardless of performance.

Attention Task 4

This task was identical to the prior task except that cue duration also varied pseudo-randomly across trials. Again, pre-cue delay varied between 0, 2, and 4-s, and the cue duration now varied between 0.8, 1.0, and 1.4-s. This task placed a greater demand on sustained attention because the cue duration was often very brief. Mice were tested for 10 sessions, regardless of performance.

Attention Task 5

This task was identical to the prior task except that the pre-cue delay varied between 0, 4, and 8-s, and the cue duration varied between 0.4, 0.8, and 1.2-s. This task further increased demand on inhibitory control and sustained attention. Mice were tested for 12 sessions, regardless of performance.

Tissue preparation and immunohistochemistry

Immediately following the completion of behavioral testing (~17 months of age) mice were deeply anesthetized by intraperitoneal injection of a ketamine (83 mg/kg)/xylazine (13 mg/kg) solution, then perfused transcardially with 4% paraformaldehyde (50 mL) in phosphate buffer (PB; 0.1M; pH = 7.4). Brains were removed and placed in the same fixative at 4 °C for 24 h, then immersed in a 30% sucrose solution until sectioning. Brains were cut on a freezing, sliding microtome in the coronal plane at 40-μm thickness into six series and stored at 4 °C in a cryoprotectant solution (30% glycerol, 30% ethylene glycol, in 0.1M PB) until processing.

Immunohistochemistry was performed as previously described (Ash et al., 2014; Kelley et al., 2014a, 2014b, 2016). Sections were singly immunolabeled with either a goat polyclonal antibody for ChAT (1:1000 dilution, Millipore, Billerica, MA), or a rabbit polyclonal antibody against p75^{NTR} (1:2000 dilution, Millipore). Tissue was washed in 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 rinsed 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 plate. 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). 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), 1% nickel (II) ammonium sulfate hexahydrate, and 0.0015% H_2O_2. Sections were washed in acetate-imidazole buffer to terminate reactions, mounted onto chrome-alum-submersed 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. Antibody penetration was determined by evaluating tissue through z-stacks using the stereology system as previously reported (Kelley et al., 2014a, 2014b). Control sections were processed using the same procedures as above with the exception of incubation with the primary antibody.

Morphometric Analysis

The BFCN subregions examined were the MSN and NBM/SI (Kelley et al., 2014a, 2014b). Estimation of ChAT- and p75^{NTR}-immunoreactive (ir) neuron numbers were derived using the optical fractionator, a stereology system that pairs the optical disector probe (a three-dimensional counting space) with a two-dimensional grid that provides an unbiased random start and systematic interval sampling of the region of interest. All analyses were conducted using Stereo Investigator software (version 9.14.5 32-bit, MicroBrightField, Inc., Williston,

VT) coupled to a Nikon Optiphot-2 microscope, as reported previously (Ash et al., 2014; Kelley et al., 2014a, 2014b). Values are presented as estimate per brain derived from a sampling of the region of interest bilaterally across a 1/6 series for each marker (X60 n.a., 1.40, 50×50 μm counting frame, 151×151 μm grid size, 10 μm disector height). Tissue thickness was measured at every site that contained cells and the reciprocal for (disector height) / (mean measured thickness) was used for reported numbers and statistical analyses. 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 $μm^3$. Calculation was performed for each animal, prior to group averages.

BFCN volume was measured using a 5-ray nucleator probe for an average of 60 cells per stain, per region, per animal (X60 oil-immersion lens n.a. 1.40) using random sampling across rostrocaudal and dorsoventral axes derived with the optical fractionator. The nucleator involves taking five measurements from an approximate center of the cell to the perimeter of the cell in one plane (< 1.0 μm z-axis) of section (Gundersen, 1988). The probe derives an average radius for each cell and volume was calculated from this value using a weighted geometric formulae (shape assumption spheroid).

Statistical Analyses

Statistical analyses were performed using the Statistical Analysis System (version 9.3; SAS Institute, Cary, NC). Attention task performance measures were analyzed using PROC GLM when trial conditions did not vary and performance was stable across testing sessions, or PROC GLIMMIX when the task involved a time (learning) component or variable trial conditions. PROC GLIMMIX is a generalized linear mixed models procedure for conducting repeated measures analyses. The dependent measures included: trials to criterion, errors to criterion, percentage of correct responses, percentage of inaccurate responses, percentage of premature responses, and percentage of omission errors. All error types factor into the percentage of correct responses, which provides a global index of performance. Fixed factors for the models included genotype, maternal diet, session block, stimulus delay, stimulus duration, and the outcome of the previous trial. Percentage of correct responses was analyzed as a function of the outcome of the previous trial based on our prior finding that Ts65Dn mice exhibit stress-induced stereotypical behavior after committing an error (Driscoll et al., 2004). Morphometric analyses were conducted using 2 X 2 ANOVAs. The dependent measures included the number, density, and volume of ChAT- and $p75^{NTR}$-ir cells within the MSN and NBM/SI. A non-parametric Spearman rank correlation was used to assess the relationship between task performance and morphometric indices of BFCNs. These analyses included all mice for which both behavioral and morphometric indices were available. The alpha level was set at 0.05 for all analyses.

Results

Several task variables had highly significant effects on performance for all mice: percentage correct decreased as the duration of the pre-cue delay increased ($p < 0.0001$); percentage correct decreased as the cue duration decreased ($p < 0.0001$); and percentage correct

decreased on trials following an error ($p < 0.001$). These variables are only described when there was a significant interaction with genotype and/or maternal diet.

Initial visual discrimination task

There was no significant effect of genotype on the number of trials to criterion [F (1, 48) = 2.33, $p = 0.13$] or errors to criterion [F (1, 48) = 1.44, $p = 0.23$]. There was also no significant effect of maternal diet on the number of trials to criterion [F (1, 48) = 1.23, $p = 0.27$] or errors to criterion [F (1, 48) = 0.95, $p = 0.33$].

Attention Task 1 (2-s cue duration; no pre-cue delay)

Analysis of percentage correct revealed a significant main effect of genotype [F (1, 46) = 13.42, $p < 0.001$]. Ts65Dn mice performed significantly worse than 2N mice (data not shown). There was no significant effect of maternal diet, nor was there a significant interaction of genotype and maternal diet.

We observed a significant main effect of genotype on percentage of omissions [F (1, 46) = 11.90, $p = 0.001$], indicating that deficits in Ts65Dn mice were driven by an increase in this type of error. There was no effect of genotype on percentage of inaccurate responses. However, there was a significant main effect of maternal diet [F (1, 46) = 6.41, $p = 0.01$], with choline-supplemented mice making fewer inaccurate responses than their unsupplemented counterparts.

Attention Task 2 (1-s cue duration; no pre-cue delay)

Analysis of percentage correct revealed a significant main effect of genotype [F (1, 48) = 31.69, $p < 0.001$]. There was also a significant main effect of maternal diet [F (1, 48) = 4.85, $p = 0.03$]. Ts65Dn mice performed significantly worse than the 2N mice, and choline-supplemented mice performed significantly better than their unsupplemented counterparts (Figure 1A). There was no significant interaction of genotype and maternal diet.

As in task 1, there was a significant main effect of genotype on percentage of omissions [F (1, 48) = 18.65, $p < 0.0001$], revealing that deficits in Ts65Dn mice were driven by an increase in these errors. Also as seen in task 1, there was a significant main effect of maternal diet on percentage of inaccurate responses [F (1, 48) = 11.94, $p = 0.001$]. Improved performance in choline-supplemented mice was due to a lower percentage of inaccurate responses compared to the unsupplemented mice (Figure 1B).

Attention Task 3 (1-s cue duration; 0, 2, or 4-s pre-cue delay)

Analysis of percentage correct revealed a significant main effect of session block [F (5, 368) = 151.72, $p < 0.001$], reflecting the improvement of all groups across session blocks. A significant main effect of genotype [F (1, 51) = 17.48, $p < 0.001$] was also observed, as well as a significant interaction of genotype X block [F (5, 368) = 11.86, $p < 0.001$]. Ts65Dn mice did not differ from 2N mice at blocks 1 or 2, but performed significantly more poorly during blocks 3–6 (Figure 2A). There was no significant effect of maternal diet, nor was there a significant interaction of genotype and maternal diet.

Analysis of percentage of premature responses revealed a significant main effect of session block [F (5, 211) = 113.83, $p < 0.001$]. All groups initially made a high percentage of premature responses, but improved across session blocks as they learned to wait for the cue to be presented (Figure 2B). There was a significant interaction of genotype X block [F (5, 211) = 9.62, $p < 0.001$]. Ts65Dn mice made a lower percentage of premature responses than 2N mice during session block 1, but a significantly higher percentage of premature responses during session blocks 3–6 (Figure 2B). There also was a significant interaction of maternal diet X block [F (5, 211) = 6.93, $p < 0.001$]. Choline supplemented mice made a lower percentage of premature responses than unsupplemented mice during session block 2; maternal diet did not influence this measure for other session blocks (Figure 2B).

Ts65Dn mice made more omission errors than 2N mice, as indicated by a significant main effect of genotype [F (1, 54) = 13.22, $p < 0.001$]. Analysis of the percentage of inaccurate responses revealed significant main effects of genotype [F (1, 54) = 4.46, $p = 0.04$] and maternal diet [F (1, 54) = 3.89, $p = 0.05$]. Ts65Dn mice made a significantly higher percentage of inaccurate responses than 2N mice and, as in task 2, choline-supplemented mice made a significantly lower percentage of inaccurate responses than unsupplemented mice (Figure 2C).

Attention Task 4 (0.8, 1.0, or 1.4-s cue duration; 0, 2, or 4-s pre-cue delay)

Analysis of percentage correct revealed a significant main effect of genotype [F (1, 56) = 36.79, $p < 0.001$]. Across all pre-cue delay and cue duration conditions, Ts65Dn mice performed more poorly than the 2N mice. We observed a significant genotype X delay interaction [F (2, 186) = 7.29, $p < 0.001$]. Ts65Dn mice were impaired relative to the 2N mice at all delays, but the pattern of performance across delays varied by genotype. Specifically, the performance of Ts65Dn mice declined significantly on trials with a 2-s delay, and declined further on trials with a 4-s delay; in contrast, the performance of the 2N mice was similar on trials with 0-s and 2-s delays, and declined only on trials with a 4-s delay (Figure 3). There was no significant effect of maternal diet, nor was there a significant interaction of genotype and maternal diet.

Deficits in Ts65Dn mice were driven by increases in all three error types. There were significant main effects of genotype on percentage of premature responses [F (1, 52) = 4.58, $p = 0.04$], percentage of omissions [F (1, 51) = 21.11, $p < 0.0001$], and percentage of inaccurate responses [F (1, 49) = 3.97, $p = 0.05$]. There was no significant effect of maternal diet on any error type.

Attention Task 5 (0.4, 0.8, or 1.2-s cue duration; 0, 4, or 8-s pre-cue delay)

Analysis of percentage correct revealed a significant main effect of genotype [F (1, 37) = 21.71, $p < 0.001$] and a significant genotype X delay interaction [F (2, 81) = 6.41, $p = 0.003$]. Ts65Dn mice performed worse than 2N mice on trials with either a 0-s or 4-s pre-cue delay, but the groups did not differ on trials with an 8-s delay (Figure 4A). There was also a significant 4-way interaction of genotype X maternal diet X delay X previous trial outcome [F (2, 691) = 4.24, $p = 0.01$]. On trials that both followed an error and involved immediate cue presentation (0-s delay), the choline-supplemented Ts65Dn mice performed

significantly better than their unsupplemented counterparts, and did not differ from the unsupplemented 2N mice (Figure 4B); in contrast, maternal diet did not affect performance of the 2N mice for this condition.

Again, deficits in Ts65Dn mice were due to increases in all three types of errors. There were significant main effects of genotype on the percentage of premature responses [F (1, 78) = 3.83, $p = 0.05$] and the percentage of omissions [F (1, 44) = 4.78, $p = 0.03$], although neither of these errors were affected by maternal diet. Analysis of the percentage of inaccurate responses revealed a significant main effect of genotype [F (1, 27) = 10.74, $p = 0.003$], as well as a significant interaction of genotype X maternal diet X delay X cue duration [F (4, 726) = 2.31, $p = 0.05$]. MCS significantly reduced the percentage of inaccurate responses of the 2N mice under specific trial conditions: choline-supplemented 2N mice made a lower percentage of inaccurate responses than their unsupplemented counterparts on trials in which the briefest cue (0.4-s) was presented following either a 0-s pre-cue delay (Figure 5A) or a 4-s pre-cue delay (Figure 5B), and on trials in which a slightly longer cue (0.8-s) was presented following an 8-s pre-cue delay (Figure 5C).

BFCN quantitative morphometry

NBM/SI ChAT-ir neuron number was significantly greater for the Ts65Dn mice compared to 2N mice [F (1, 54) = 6.93, $p = 0.01$] (Figure 6A), but neuron density did not differ by group (Figure 6B). Volume of ChAT-ir neurons was significantly increased in Ts65Dn mice compared to 2N mice [F (1, 54) = 4.84, $p = 0.03$] (Figure 6C). The number of $p75^{NTR}$-ir neurons in the NBM/SI did not differ by group (Figure 6D), but the density of neurons in this region was significantly reduced in the Ts65Dn mice relative to 2N mice [F (1, 53) = 6.36, $p = 0.01$] (Figure 6E). As observed in the ChAT-ir neurons, the volume of $p75^{NTR}$-ir neurons was significantly larger for the Ts65Dn mice compared to 2N mice [F (1, 53) = 6.32, $p = 0.01$] (Figure 6F). None of these measures of NBM/SI morphology were affected by maternal diet.

In the MSN, ChAT-ir neuron number was significantly decreased in Ts65Dn mice compared to 2N mice [F (1, 48) = 8.39, $p = 0.01$] (Figure 7A), as was density of these neurons [F (1, 48) = 29.77, $p < 0.001$] (Figure 7B). Volume of ChAT-ir neurons in the MSN was significantly increased in Ts65Dn mice compared to 2N mice [F (1, 48) = 7.89, $p = 0.01$] (Figure 7C). The number of $p75^{NTR}$-ir neurons in this region did not vary by genotype (Figure 7D), but density of these neurons was decreased in the Ts65Dn mice relative to 2N mice [F (1, 52) = 6.02, $p = 0.02$] (Figure 7E). As observed in the ChAT-ir neurons (and as in the NBM/SI), the volume of $p75^{NTR}$-ir neurons in the MSN was increased in the Ts65Dn mice compared to 2N mice [F (1, 52) = 13.3, $p < 0.001$] (Figure 7F). None of these measures of MSN morphology were affected by maternal diet.

Correlations between behavior and BFCN quantitative morphometry

Correlational analyses revealed significant *negative* associations between NBM/SI ChAT-ir neuron volume and percentage correct on task 2 [$r_s = -0.43$, $p = 0.002$] (Figure 8A), task 3 [$r_s = -0.28$, $p = 0.04$] (Figure 8B), and task 4 [$r_s = -0.31$, $p = 0.03$] (Figure 8C); i.e., large neuron volume was associated with poor performance. We also observed significant *negative*

associations between MSN ChAT-ir neuron volume and percentage correct on task 2 [$r_s =$ −0.37, $p = 0.01$] (Figure 8D), task 3 [$r_s =$ −0.51, $p = 0.0003$] (Figure 8E), and task 4 [$r_s =$ −0.38, $p = 0.009$] (Figure 8F). Similar to findings with ChAT-ir neuron volume, parallel correlations were observed between p75^{NTR}-ir neuron volume and task performance (data not shown).

Lastly, we observed significant *positive* associations between MSN ChAT-ir neuron density and percentage correct on task 2 [$r_s = 0.61$, $p < 0.0001$] (Figure 9A), task 3 [$r_s = 0.29$, $p = 0.04$] (Figure 9B), and task 4 [$r_s = 0.47$, $p = 0.001$] (Figure 9C), indicating that lower neuron density was associated with poorer task performance.

Discussion

Effects of trisomy and MCS on learning and attention

The rate of learning the initial visual discrimination task did not differ between Ts65Dn and 2N mice, and was not affected by maternal diet. This indicates that neither trisomy nor MCS altered motivation, visual acuity, nor motor functions needed to perform these tasks. Trisomic mice were impaired when the duration of the visual cue was reduced, which increased the demands on attention. Deficits were observed across all attention tasks, and were primarily due to an increased incidence of omission errors and inaccurate responses. Based on a prior study, which analyzed video recordings of mice performing these tasks (Driscoll et al., 2004), omission errors typically occur when mice completely miss the cue, whereas inaccurate responses tend to occur when mice are momentarily off-task, notice that the visual cue was presented, but are unsure from which response port the cue emanated. Thus, the increased incidence of these two error types implicates attentional dysfunction in the trisomic mice. Finally, the Ts65Dn mice also committed a higher percentage of premature responses than 2N mice on tasks with pre-cue delays, indicating deficient inhibitory control.

A significant benefit of MCS was observed for both Ts65Dn and 2N offspring. On the first two attention tasks (brief cue, but no delay prior to cue presentation), choline-supplemented mice performed better than their unsupplemented counterparts. In addition, when the pre-cue delays were first introduced, choline-supplemented mice of both genotypes learned to wait for the cue more quickly than their unsupplemented counterparts. Observed benefits of MCS are in agreement with results from our prior study (Moon et al., 2010), although the benefit seen for the trisomic mice was less pronounced. A likely reason is that mice were tested at an older age (12–17 mo.) than in our earlier study (6–10 mo.) (Moon et al., 2010). This suggests that MCS slows, but does not prevent, age-related neuropathological processes.

We added a new attention task, more challenging than used in prior studies. Task 5, which included the longest pre-cue delays and shortest cue durations, revealed benefits of MCS for both 2N and Ts65Dn mice, albeit in different domains. Choline-supplemented 2N mice made a lower percentage of inaccurate responses on specific trial conditions with brief cue durations, suggesting that they more vigilantly attended to the response ports. MCS also was beneficial for Ts65Dn offspring in this task, but the nature of the benefit was different. Choline-supplemented Ts65Dn mice performed significantly better than their

unsupplemented counterparts specifically on trials that both followed an error and in which there was no pre-cue delay. This is the condition predicted to be impacted negatively by committing an error on the previous trial, because less time had elapsed to allow the frustration of the error to dissipate. Under these conditions, choline-supplemented Ts65Dn mice were significantly less disrupted by an error on the prior trial than their unsupplemented counterparts. This pattern of effects supports the observations of Moon et al. (2010), where Ts65Dn mice were more likely to make an error on trials that followed an error, because their emotional reactions to committing an error were more excessive or less well-regulated. MCS was found to partially normalize these emotional reactions. These results are also consistent with a report suggesting improved emotion regulation in rats supplemented with choline prenatally (Cheng et al., 2008).

Overall, the pattern of results indicates that the cognitive benefits derived by MCS in these tasks are in the domains of learning, attention, and emotion. These findings add to a growing body of literature demonstrating that supplementing the maternal diet with increased choline produces lifelong improvements in attentional function and spatial cognition for normal (Meck et al., 1988, 1989; Meck and Williams, 1997, 1999; Mohler et al., 2001; Moon et al., 2010; Tees and Mohammedi, 1999; Williams et al., 1998) and Ts65Dn (Ash et al., 2014; Moon et al., 2010; Velazquez et al., 2013) offspring.

BFCN quantitative morphometry and the relationship to cognitive function

A major goal of the present study was to characterize the effects of the Ts65Dn trisomy on morphology of cholinergic neurons in the NBM/SI, and determine whether any observed changes correlate with attentional function. Several research groups have investigated the BFCN system in Ts65Dn mice, but nearly all have focused on the MSN.

Numerous previous reports have shown that Ts65Dn mice exhibit a reduction in MSN cholinergic neuron number compared to 2N mice (Contestabile et al., 2006; Cooper et al., 2001; Granholm et al., 2000; Holtzman et al., 1996; Hunter et al., 2004; Salehi et al., 2006; Seo and Isacson, 2005). We have replicated this effect in Ts65Dn mice in the current report, as well as in siblings assessed for spatial memory (Ash et al., 2014). In contrast, the present study revealed, for the first time, that Ts65Dn mice exhibit a significantly *greater* number of NBM/SI cholinergic neurons than their 2N counterparts. A recent study from our lab describing changes in BFCN morphology as a function of age provides insight into the basis of this effect (Powers et al., 2016). We observed that the number of NBM/SI ChAT-ir neurons decreases with age in 2N, but not Ts65Dn mice, resulting in a greater neuron number in aged trisomic mice compared to aged disomic controls. This abnormality may represent a compensatory mechanism in Ts65Dn mice in response to cholinergic dysfunction. It is noteworthy that the effects of the trisomy on BFCN number and density were qualitatively different between the NBM/SI and MSN within this single cohort of animals. These findings caution against generalizing effects of this trisomy across BFCN subfields.

The present study also revealed that the volume of both ChAT- and p75[NTR]-ir neurons within the NBM/SI and the MSN was significantly *greater* for Ts65Dn relative to 2N mice. A recent observation reported by our group has shown that the soma size of cholinergic

neurons in both the NBM/SI and MSN decreased with age in 2N mice, but not in Ts65Dn mice (Powers et al., 2016). We postulate that this failure of the cholinergic system to undergo normal aging-related change may reflect a compensatory mechanism in the aging trisomic brain in response to progressive cholinergic signaling deficits.

Correlational analyses linking BFCN morphology and attentional function provided new insights into the basis of attention performance in these tasks, and by inference, the basis of the attentional dysfunction in Ts65Dn mice. Specifically, these correlational analyses revealed that BFCN volume in both the NBM/SI and MSN was *negatively* associated with attention task performance. This suggests either that large neurons do not function optimally, or that large neurons are a symptom of a separate disturbance in the system, which itself is functionally related to attention. In addition, BFCN density in the MSN correlated *positively* with attention task performance. Similarly, in the siblings of the present subjects, tested in the water maze (Ash et al., 2014), BFCN density in the MSN correlated negatively with errors in the water maze; i.e., increased density was associated with improved spatial cognition. Although it seems plausible that reduced density of MSN cholinergic neurons may be causally related to spatial maze deficits in Ts65Dn mice, it seems unlikely to underlie attentional dysfunction. Rather, it seems more plausible that other age-related neurodegeneration, which itself correlates with MSN neurodegeneration, is associated with attentional dysfunction.

MCS did not affect MSN cholinergic neuron morphometry in either genotype. This is in contrast to the results seen in the offspring tested in the radial arm water maze, where MCS prevented a decrease in number and density of MSN cholinergic neurons seen in the trisomic offspring of unsupplemented dams (Ash et al., 2014). The reasons for these discrepant results are unclear, but likely reflect substantial differences in behavioral testing procedures, food restriction regimen, and social isolation experienced by the two cohorts, factors which may have interacted with the effects of MCS. The training on this series of attention tasks involved appetitive motivation, and was lengthy, spanning 5 months. In contrast, the other cohort was tested in a radial arm water maze, which involved aversive motivation and was shorter, spanning less than one month. In addition to the different behavioral and cognitive demands, the series of attention tasks used in the present study required a more stringent and longer period of food restriction than the water maze procedure. A growing literature suggests that calorie restriction itself can be neuroprotective in mouse models of neurodegenerative disorders (Contestabile, 2009; Schafer et al., 2015a, 2015b), and thus may have interacted with the effects of MCS and/or the trisomy. BFCN morphometry may also have been influenced by social isolation. All mice were singly-housed during behavioral testing, but because the attention testing took place over a much longer period of time than the water maze testing, the social isolation experienced by these animals was considerably longer than that experienced by the radial arm water maze animals. It will be important for future studies to further explore factors which may interact with the effects of MCS on BFCN morphometry.

The present study contributed novel information about NBM/SI cholinergic neuron morphology in Ts65Dn mice, and provided a test of the hypothesis that the improved attention seen in the offspring of choline-supplemented dams is due to effects on NBM/SI

neurons. However, in light of the present results, further work is required to elucidate the underlying mechanism of this beneficial attentional effect of MCS. In this regard, it is possible that MCS affects the noradrenergic neurons in the locus coeruleus, which innervate the cortex and hippocampus (Counts and Mufson, 2010), modulate memory, attention, and vigilance (Samuels and Szabadi, 2008), and degenerate with age in Ts65Dn mice (Lockrow et al., 2011).

Therapeutic recommendation for increased choline intake during development

Our group has demonstrated that supplementing the maternal diet with additional choline has numerous beneficial effects for Ts65Dn and 2N offspring, including improvements in attention, emotion regulation, spatial memory, hippocampal neurogenesis, and protection of cholinergic neurons in the MSN (Ash et al., 2014; Moon et al., 2010; Strupp et al., 2016; Velazquez et al., 2013). Choline supply is critical for the developing brain because it is a precursor of acetylcholine, a key neurotransmitter for regulating neuronal proliferation, differentiation, migration, maturation, plasticity, and survival, as well as synapse formation (Abreu-Villaca et al., 2011; Albright et al., 1999; Blusztajn, 1998; Cermak et al., 1999; Zeisel, 2011). Choline is also the precursor of phosphatidylcholine and sphingomyelin, principal components of neuronal and other cellular membranes required for signal transduction, brain development and fetal growth (Blusztajn, 1998). Moreover, choline is the primary dietary source of methyl groups in humans, and maternal dietary choline intake can exert lasting effects on offspring gene expression via modulation of DNA and histone methylation (Blusztajn et al., 2012; Jiang et al., 2012). Collectively, growing evidence suggests that increasing choline intake may be an advisable therapeutic recommendation for pregnant and lactating women, a dietary change that may not only improve cognitive functioning and reduce aging-related decline in the population at large, but also minimize cognitive dysfunction in offspring with DS. Notably, because this dietary advice could be given to all pregnant women, this type of DS intervention could start at the earliest stages of development, and include women who do not know they are carrying a fetus with DS.

Acknowledgments

Abbreviations

AD	Alzheimer's disease
BFCNs	basal forebrain cholinergic neurons
ChAT	choline acetyltransferase
DS	Down syndrome
MCS	maternal choline supplementation
MSN	medial septal nucleus

| **NBM/SI** | nucleus basalis of Meynert/substantia innominata |
| **p75^{NTR}** | pan-neurotrophin receptor p75^{NTR} |

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Highlights

- Ts65Dn mice demonstrated impaired learning and attention, which were ameliorated by maternal choline supplementation (MCS).

- MCS improved attention in both Ts65Dn and 2N offspring, but did not alter morphology of cholinergic neurons in the NBM/SI.

- Ts65Dn mice exhibited an *increased* number of BFCNs in the NBM/SI and a *decreased* number of BFCNs in the MSN.

- The soma volume of BFCNs in both basal forebrain regions was significantly larger in Ts65Dn mice, relative to 2N controls.

- Attentional performance was inversely associated with BFCN volume, and positively associated with BFCN density.
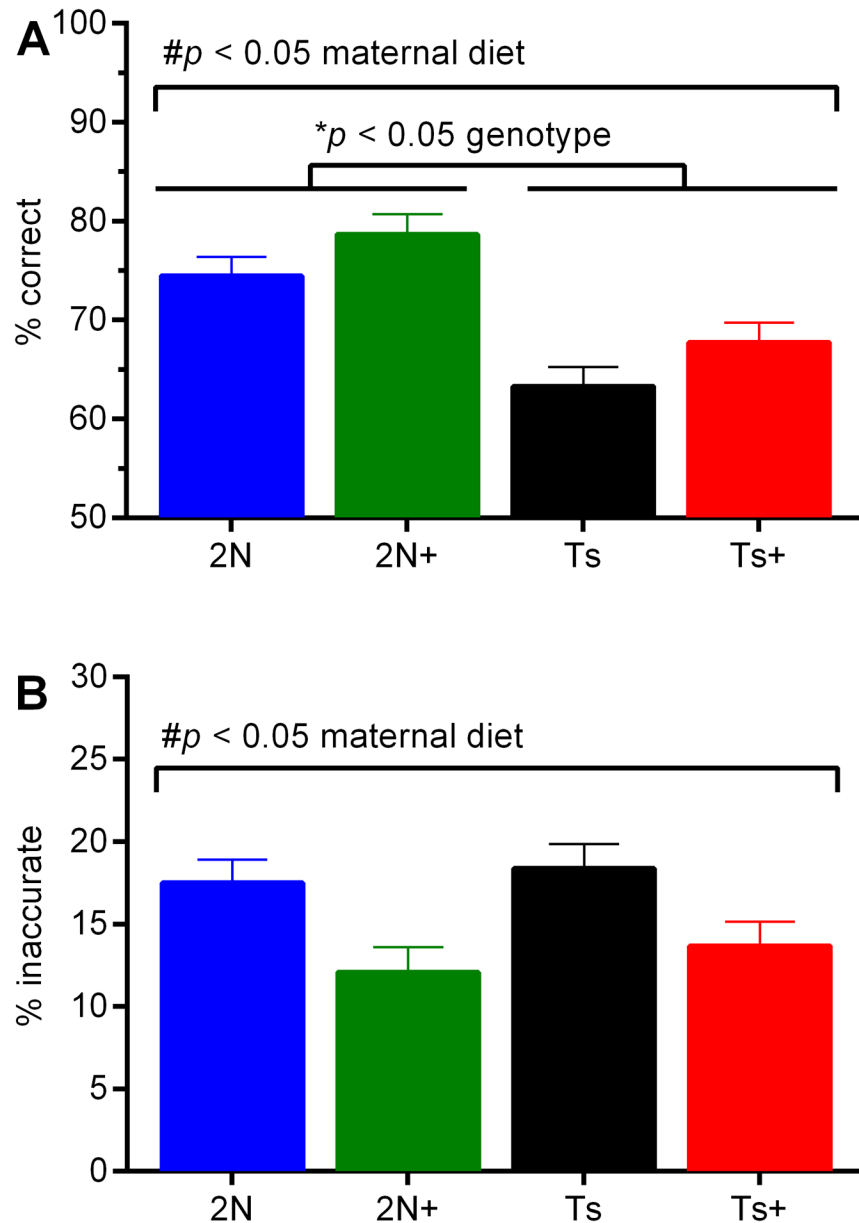
Figure 1. Attention Task 2

(A) Mean (±SEM) percentage correct across 8 sessions. Ts65Dn mice performed more poorly than 2N mice, and choline supplemented mice performed better than unsupplemented mice. **(B)** Mean (±SEM) percentage inaccurate across 8 sessions. Choline supplemented mice made fewer inaccurate responses than unsupplemented mice. $*p < 0.05$ genotype, $\#p < 0.05$ maternal diet. 2N n=14, 2N+ n=12, Ts n=13, Ts+ n=13.
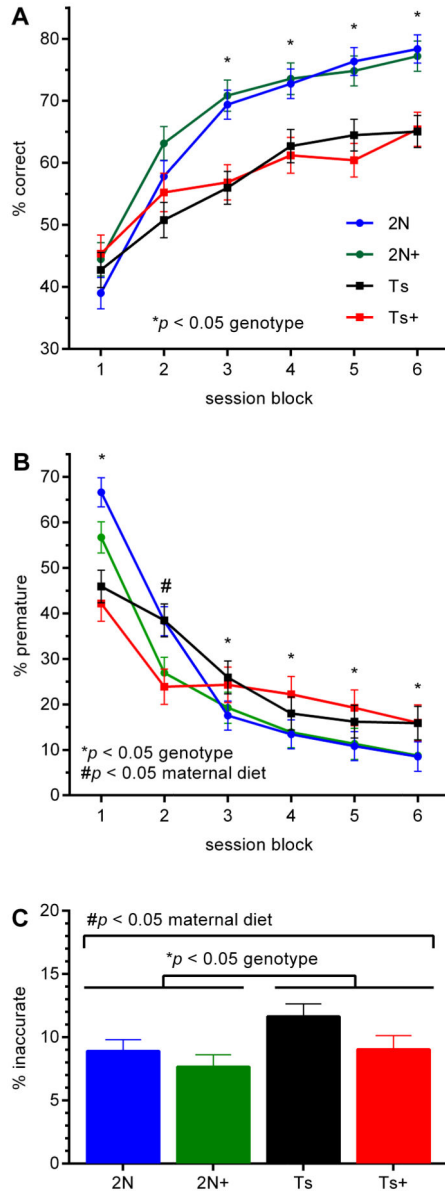
Figure 2. Attention Task 3

(A) Mean (±SEM) percentage correct across 6 blocks of 3 sessions each, averaged across all pre-cue delay conditions. All groups performed similarly in blocks 1 & 2 (as they learned to wait during the pre-cue delay), but there was a performance deficit in the Ts65Dn mice across blocks 3–6. **(B)** Mean (±SEM) percentage premature. All groups initially made many premature responses, but improved as they learned to wait for the cue. Ts65Dn mice made fewer premature responses than 2N mice during block 1, but made more premature responses than 2N mice across blocks 3–6. Both choline supplemented groups appeared to learn to wait at a faster rate than unsupplemented groups, making fewer premature responses at block 2, but a benefit of MCS was not observed in blocks 3–6. **(C)** Mean (±SEM) percentage inaccurate across all sessions. Ts65Dn mice made more inaccurate responses than 2N mice, and choline supplemented mice made fewer inaccurate responses than

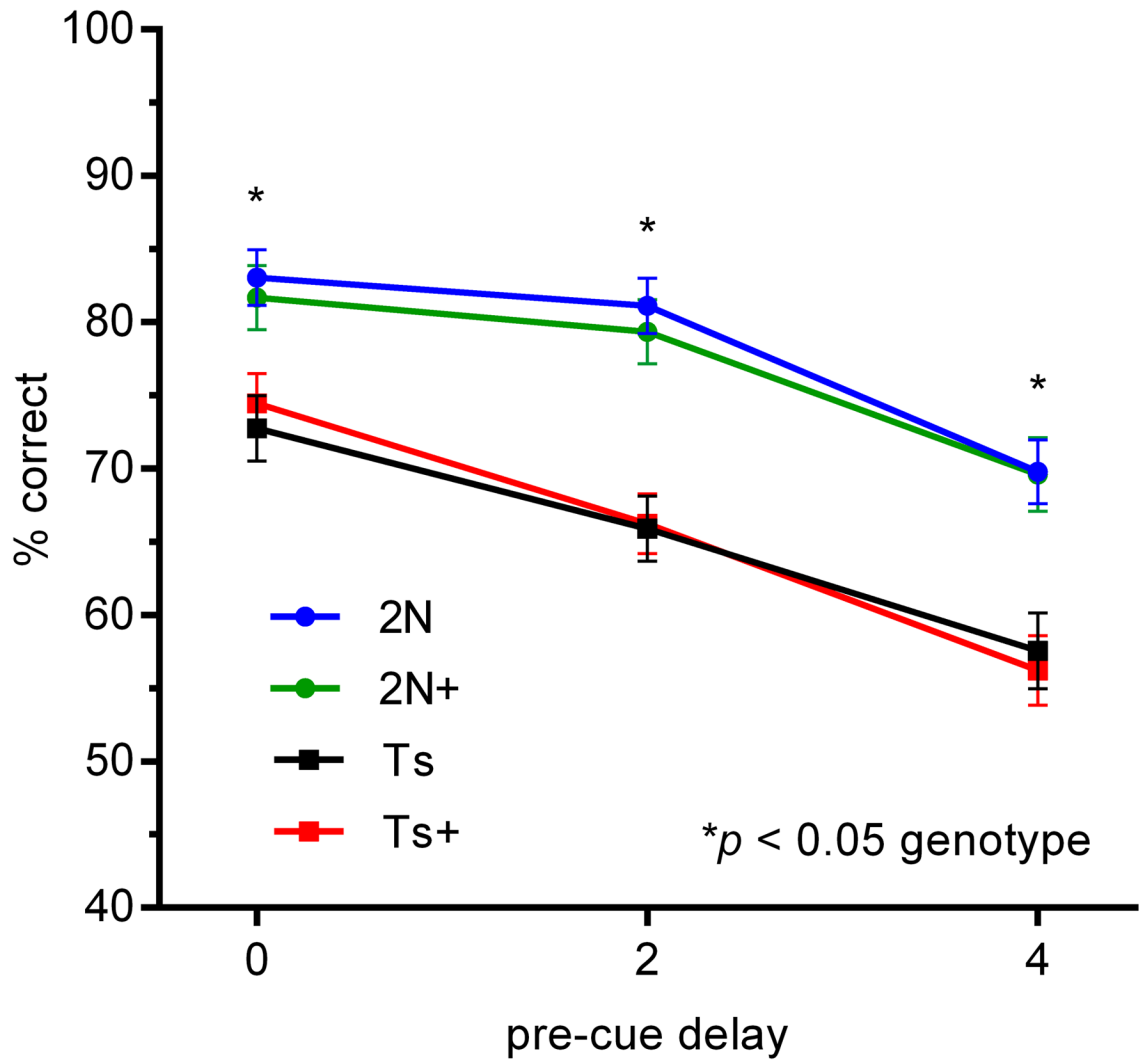unsupplemented mice. *$p < 0.05$ genotype, #$p < 0.05$ maternal diet. 2N n=16, 2N+ n=14, Ts n=13, Ts+ n=11.

Figure 3. Attention Task 4
Mean (±SEM) percentage correct across 10 sessions, as a function of precue delay. Performance of all groups declined on trials with a 4-s pre-cue delay, but a performance deficit in Ts65Dn mice was evident at all delay conditions. *$p < 0.05$ genotype. 2N n=16, 2N+ n=12, Ts n=11, Ts+ n=13.
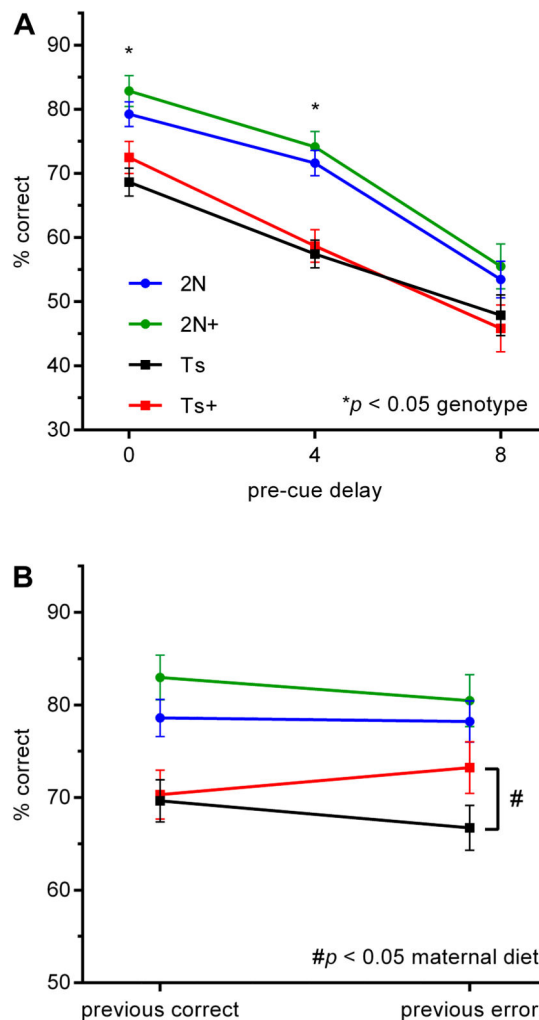
Figure 4. Attention Task 5

(A) Mean (±SEM) percentage correct across 12 sessions, as a function of pre-cue delay. Ts65Dn mice exhibited deficits on trials with a 0-s or 4-s pre-cue delay. On trials with an 8-s pre-cue delay performance declined in all groups, and there were no significant differences. **(B)** Mean (±SEM) percentage correct on trials with no pre-cue delay, following a previous trial with either a correct response or an error. There was a significant benefit of MCS in Ts65Dn mice on trials following an error. $*p < 0.05$ genotype. $\#p < 0.05$ maternal diet. 2N n=15, 2N+ n=10, Ts n=12, Ts+ n=9.
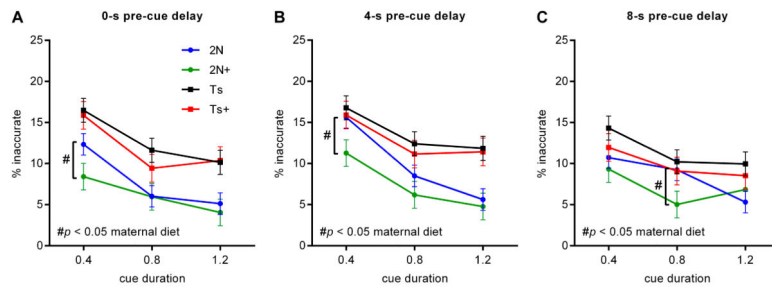
Figure 5. Attention Task 5

Mean (±SEM) percentage inaccurate as a function of cue duration on trials with a 0-s pre-cue delay **(A)**, trials with a 4-s pre-cue delay **(B)**, and trials with an 8-s pre-cue delay **(C)**. There was a significant benefit of MCS in 2N mice on trials with a 0-s or 4-s pre-cue delay and a 0.4-s cue duration, and on trials with an 8-s pre-cue delay and a 0.8-s cue duration. #p < 0.05 maternal diet. 2N n=15, 2N+ n=10, Ts n=12, Ts+ n=9.
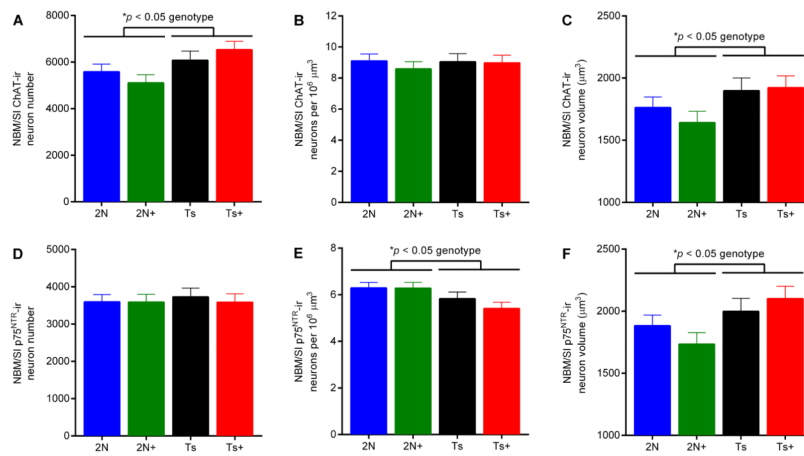
Figure 6. BFCNs in the NBM/SI

(A) ChAT-ir neuron number was significantly increased in Ts65Dn mice. **(B)** ChAT-ir neuron density did not differ by group. **(C)** ChAT-ir neuron volume was significantly increased in Ts65Dn mice. **(D)** p75^{NTR}-ir neuron number did not differ by group. **(E)** p75^{NTR}-ir neuron density was significantly decreased in Ts65Dn mice. **(F)** p75^{NTR}-ir neuron volume was significantly increased in Ts65Dn mice. $*p < 0.05$ genotype. 2N n=16, 2N+ n=14, Ts n=11, Ts+ n=13.
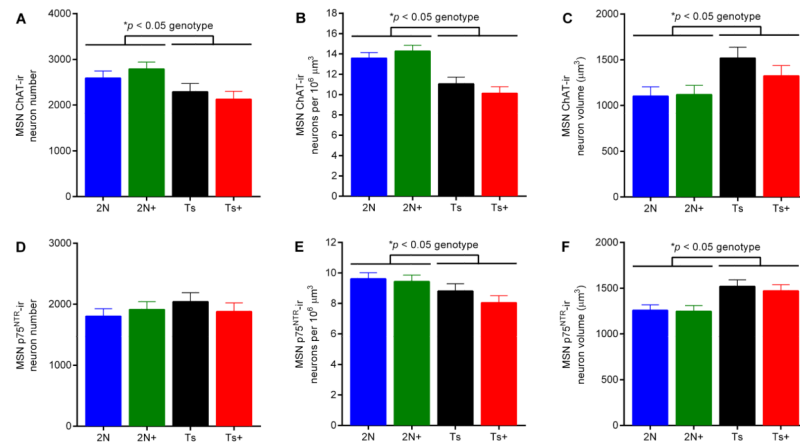
Figure 7. BFCNs in the MSN
(A) ChAT-ir neuron number was significantly decreased in Ts65Dn mice. **(B)** ChAT-ir neuron density was significantly decreased in Ts65Dn mice. **(C)** ChAT-ir neuron volume was significantly increased in Ts65Dn mice. **(D)** p75NTR-ir neuron number did not differ by group. **(E)** p75NTR-ir neuron density was significantly decreased in Ts65Dn mice. **(F)** p75NTR-ir neuron volume was significantly increased in Ts65Dn mice. $*p < 0.05$ genotype. 2N n=14, 2N+ n=14, Ts n=11, Ts+ n=12.
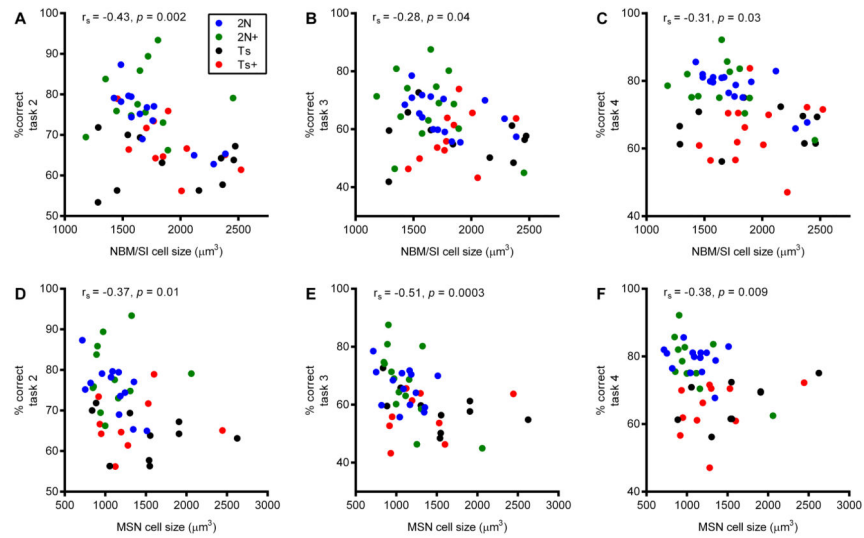
Figure 8. Correlations between attention task performance and BFCN volume
There were significant negative associations between NBM/SI ChAT-ir neuron volume and percent correct on task 2 **(A)**, task 3 **(B)**, and task 4 **(C)**. There also were significant negative associations between MSN ChAT-ir neuron volume and percent correct on task 2 **(D)**, task 3 **(E)**, and task 4 **(F)**. 2N n=13–16, 2N+ n=12–14, Ts n=9–11, Ts+ n=9–13.
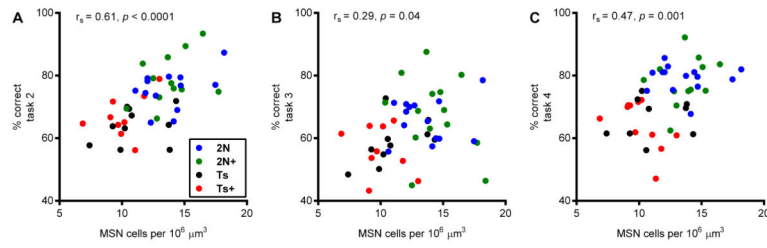
Figure 9. Correlations between attention task performance and BFCN density

There were significant positive associations between MSN ChAT-ir neuron density and percent correct on task 2 **(A)**, task 3 **(B)**, and task 4 **(C)**. 2N n=13–14, 2N+ n=12–14, Ts n=9–10, Ts+ n=9–11.