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Maternal choline supplementation improves spatial learning and adult hippocampal neurogenesis in the Ts65Dn mouse model of Down syndrome

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Abstract

In addition to intellectual disability, individuals with Down syndrome (DS) exhibit dementia by the third or fourth decade of life, due to the early onset of neuropathological changes typical of Alzheimer's disease (AD). Deficient ontogenetic neurogenesis contributes to the brain hypoplasia and hypocellularity evident in fetuses and children with DS. A murine model of DS and AD (the Ts65Dn mouse) exhibits key features of these disorders, notably deficient ontogenetic neurogenesis, degeneration of basal forebrain cholinergic neurons (BFCNs), and cognitive deficits. Adult hippocampal (HP) neurogenesis is also deficient in Ts65Dn mice and may contribute to the observed cognitive dysfunction. Herein, we demonstrate that supplementing the maternal diet with additional choline (approximately 4.5 times the amount in normal rodent chow) dramatically improved the performance of the adult trisomic offspring in a radial arm water maze task. Ts65Dn offspring of choline-supplemented dams performed significantly better than unsupplemented Ts65Dn mice. Furthermore, adult hippocampal neurogenesis was partially normalized in the maternal choline supplemented (MCS) trisomic offspring relative to their unsupplemented counterparts. A significant correlation was observed between adult hippocampal neurogenesis and performance in the water maze, suggesting that the increased neurogenesis seen in the supplemented trisomic mice contributed functionally to their improved spatial cognition.

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These findings suggest that supplementing the maternal diet with additional choline has significant translational potential for DS.

Keywords

Down Syndrome; Ts65Dn; Maternal Choline; Hippocampus; Neurogenesis; Spatial learning

Introduction

Down syndrome (DS) is the most common known cause of intellectual disability, affecting 1 in 700–1000 births. This disorder is caused by triplication of human chromosome 21(HSA21) due to nondysjunction during meiosis. In addition to intellectual disability, individuals with DS generally develop dementia by the third decade of life (Lai and Williams, 1989; Mann, 1988; Visser et al., 1997; Wisniewski et al., 1985a; Wisniewski et al., 1985b) due to the onset of Alzheimer's disease (AD)-like neuropathology, including atrophy of basal forebrain cholinergic neurons (BFCNs) (Isacson et al., 2002; Sendera et al., 2000; Whitehouse et al., 1982), and formation of neuritic plaques and neurofibrillary tangles (Wisniewski et al., 1985a).

Currently there are no clinically approved treatments for either intellectual disability or dementia in DS. The development of a mouse model of DS provides a tool to investigate the pathogenic process(es) underlying this disorder and consequently provide effective therapies. A segmental trisomy mouse model of DS, the Ts65Dn mouse (Davisson, et al., 1990; Holtzman et al., 1996), is trisomic for the distal portion of mouse chromosome 16 (MMU16), which contains approximately 94 genes orthologous to those on HSA21 (Mural et al., 2002; Patterson and Costa, 2005). Ts65Dn mice survive to adulthood and exhibit many morphological, biochemical, and transcriptional changes seen in the human disorder (Antonarakis et al., 2001; Capone, 2001; Davisson, et al., 1990; Davisson et al., 1993; Holtzman et al., 1996; Reeves et al., 1995). Notably, similar to humans with DS, these mice exhibit pronounced impairments in functions modulated by BFCN projections to the neocortex (e.g., attention; Driscoll et al., 2004; Moon et al., 2010) and hippocampus (e.g., explicit memory function; Hyde et al., 2001a,b). These cognitive deficits are seen early in life (Bianchi et al., 2010a; Guidi et al. 2011), and become more pronounced in adulthood, coincident with degeneration of BFCNs (Granholt et al., 2000; Holtzman et al., 1992; Holtzman et al., 1996; Hyde and Crnic, 2001a) and increased activation of microglia (Hunter et al., 2003).

A factor that likely contributes to the aberrant brain development and cognitive dysfunction in DS is impaired ontogenetic neurogenesis, demonstrated in humans with DS (Rachidi and Lopes, 2008) and Ts65Dn mice (Bianchi et al., 2010a). Deficient adult neurogenesis has also been demonstrated in the hippocampus (Chakrabarti et al., 2011; Clark et al., 2006; Llorens-Martin et al., 2010) and subventricular zone (Bianchi et al., 2010a,b; Chakrabarti et al., 2011) in Ts65Dn mice, likely contributing to dysfunction in spatial or declarative memory (Abrous et al., 2008; Aimone et al., 2006; Leuner et al., 2006; Lledo et al., 2006; Madsen, et

al., 2000; Shors et al., 2001, 2002). These findings suggest that treatments which restore neurogenesis will also improve brain development and cognitive function in DS.

A putative treatment for restoring neurogenesis and cognitive function in DS is to supplement the maternal diet with additional choline. Maternal choline supplementation (MCS) has been shown to improve learning, attention, and affect regulation in adult Ts65Dn offspring (Moon et al., 2010; Powers et al., 2011). Similar effects have been reported in normal rodents born to choline-supplemented dams (Cheng et al., 2008; Glenn et al., 2007, McCann et al., 2006, Meck et al., 1988; Meck et al., 1999; Meck and Williams, 2003; Mohler et al., 2001; Moon et al., 2010; Powers et al., 2011; Wong-Goodrich et al., 2008; Zeisel, 2000;). Furthermore, MCS enhances adult hippocampal neurogenesis in normal rats (Glenn et al., 2007), suggesting that this same intervention would improve neurogenesis in the Ts65Dn mouse (Bianchi et al., 2010a,b; Chakrabarti et al., 2011; Clark et al., 2006; Llorens-Martin et al., 2010). Therefore, the present study tested the hypothesis that supplementing the maternal diet with additional choline during pregnancy and lactation increases hippocampal neurogenesis and improves spatial learning of the adult trisomic offspring.

Methods

Subjects

Breeder pairs of mice (Ts65Dn female and C57Bl/6J Eicher $\times$ C3H/HeSnJ F1 male) were purchased from Jackson Laboratories (Bar Harbor, ME) and mated at Cornell University, Ithaca, N.Y. Breeder pairs were randomly assigned to receive one of two concentrations of choline chloride in the diet (1.1 and 5.0 g/kg, respectively; Dyets; Bethlehem, PA), similar to previous studies reporting lasting beneficial cognitive effects of maternal choline supplementation (Meck and Williams, 1999, 2003; Meck et al., 2007). These two diets (normal choline and choline supplemented) were provided to the dams at the time that the males and females were paired. The lower concentration of choline chloride (1.1 g/kg) is the standard concentration of choline chloride found in rodent diets, and is currently considered to provide “adequate” choline intake (Meck et al., 2007). The choline intake of the choline-supplemented dams (those in the group assigned to the diet containing 5.0 g choline/kg diet) is approximately 4.5 times the amount of choline consumed by the dams in the “control-choline” group), within the range of dietary variation observed in the human population (Detopoulou et al., 2008). These two levels of maternal choline intake continued until the pups were weaned at postnatal day (PND) PND21. Food intake of pregnant dams maintained on these two diets has not revealed an influence of the choline content (e.g., Wong-Goodrich et al., 2008).

At weaning (PND 21), tissue was obtained from ear punches and genotyped, at Jackson laboratories (Bar Harbor, ME), for the presence of the extra chromosome (HSA21) by quantitative polymerase chain reaction (qPCR) and for amplification of the viral insert in the Pdeb6b gene that leads to retinal degeneration and eventual blindness. Mice homozygous for the Pdeb6b mutation were excluded from the study. Whenever possible, one trisomic and one normal disomic (2N) male pup were selected from each litter to participate in the behavioral testing.

After weaning, all pups were maintained on a diet containing standard choline levels (1.1 g choline chloride/kg diet; Dyets # 110098; Bethlehem, PA). The daily ration was calculated to yield body weights that were approximately 90% of their free-feeding weights to prevent obesity. Pilot studies in our lab indicate that mice weighing more than 40 g had a greater tendency to float when placed in the water maze. At this time, the pups were group-housed (2–4 mice/cage) in cages equipped with various objects (plastic igloos, t-tubes, and plastic-gel bones) to lessen the environmental impoverishment of the laboratory setting. Two weeks prior to testing, the animals were moved to a room with a 12:12 reversed light cycle (lights on at 8pm) and singly housed, based on prior evidence that male mice of this strain often fight when reunited after daily behavioral testing. Since mice are nocturnal animals, we tested them during the dark portion of the day-night cycle.

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.

There were a total of 13 litters for the dams maintained on the normal choline diet (with 8 providing littermate pairs) and 13 litters for the choline-supplemented dams, with 9 providing littermate pairs. The sample sizes for the four groups were: 10 wild-type mice born to dams fed normal choline diet (2N), 11 Ts65Dn mice born to dams fed normal choline diet (Ts65Dn), 11 2N mice born to dams fed choline-supplemented diet (2N Ch+), and 11 Ts65Dn mice born to dams fed choline supplemented diet (Ts65Dn Ch +).

Assessment of spatial learning in the radial arm water maze (RAWM)

Assessing hippocampal function is challenging because Ts65Dn mice follow odor trails in the radial arm maze (Crnic, 1999) and exhibit thigmotactic behavior in the Morris water maze (Costa et al., 1999; Escorihuela et al., 1995). However, the radial arm water maze (RAWM) circumvents these problems and has been used successfully in prior studies using the Ts65Dn mouse (Bimonte-Nelson et al., 2003; Howell and Gottschall, 2012; Hunter et al., 2003; Lockrow et al., 2011) and other AD mouse models (Arendash et al., 2004).

The RAWM was configured in a pool (100 cm diameter) and contained six arms (25.5 cm high, 35 cm long, 20 cm wide) radiating from the center. This configuration created a central area of 40 cm diameter. The escape platform was a cylinder (surface 10 cm diameter, 7.5 cm tall) made of clear plastic, which was maintained 1 cm below the water surface. Water temperature was maintained at 20–22°C to prevent hypothermia but still ensure adequate motivation to find the platform. Both the inside of the pool and the escape platform were black, making the escape platform invisible. Extra-maze cues included checkered wall stripes, room furniture, beach balls, a metronome and the tester who maintained a position at the same point at the periphery of the pool throughout each session. There were a total of two testers during the course of the experiment, each testing an equal number of mice per treatment group. All behavioral testing was conducted by individuals unaware of the animals' treatment group assignment.

RAWM testing comprised three phases: (1) training, (2) hidden platform task and (3) visible platform task as described below.

Training

The training phase acclimated the mice to the maze, to swimming, and to finding the hidden platform. During this phase (termed Day 0, the day prior to the start of testing), all arms were blocked except for the start arm and the goal arm that contained the hidden platform, providing a direct escape route. This same procedure was used on the first trial of Day 1 but on all subsequent trials all arms were accessible.

Hidden platform task

The hidden platform task consisted of 5 trials per day for a total of 15 days. The hidden escape platform remained in the same location throughout testing for each animal, with the animals starting from a different arm on each of the 5 daily trials, pseudo-randomly determined. Each animal was assigned a different hidden platform location for the entirety of this task, with the goal location balanced across treatment groups. On each trial, the mice were given 60 seconds to locate the hidden platform; if the platform was not located within that period, the mouse was guided to the platform. Each animal was then given a 15 second resting period on the platform and then returned to its home cage between trials. All mice in a testing squad were given trial 1 before any mouse received trial 2, with the result that each animal had a 10–20 minute rest period between consecutive trials. This procedure prevents hypothermia, a particular concern for the trisomic mice (Iivonen et al., 2003; Stasko et al., 2006; Stasko and Costa, 2004). The mice were fed immediately following the last trial of each day.

Visible platform task

One day after completing the hidden platform task, the mice were tested on a visible platform task in the same water maze apparatus for a total of six days with five trials each day. The purpose of this task was to determine whether the groups differed in a task which does not require spatial mapping but which shares other requirements of the hidden platform task, such as swimming ability, motivation to escape from the water, ability to climb onto the platform, and visual acuity. In the visible platform task, a black curtain was placed around the water maze to obscure all extramaze cues. In this task, the location of the platform was indicated by an intramaze cue: a tall white PVC pipe affixed to the platform (3.8 cm diameter × 30.5 cm high). The platform changed location on every trial. The time to locate the visible platform, the resting period on the platform, and ITI were identical to that of the hidden platform task.

Age at testing

Behavioral testing was started when the animals were, on average, 15.4 months of age, in accordance with the hypothesis that the benefits of MCS for the Ts65Dn mice, relative to their unsupplemented counterparts, would be most evident in older animals due to effects of the intervention on both brain development and age-related neurodegeneration which begins in these mice between 4–6 months of age, and becomes more pronounced over time (Granholm et al., 2000; Holtzman et al., 1992; Holtzman et al., 1996; Hyde and Crnic, 2001a). Due to logistical constraints, it was necessary to test the animals in two consecutive

cohorts, balanced for the 4 treatment groups. As a result, the age at the start of testing ranged from 13 to 17 months (mean = 15.4 months), balanced for the four treatment groups.

Tissue Preparation

Upon completion of behavioral testing, the mice were deeply anesthetized with ketamine (85 mg/kg)/xylazine (13 mg/kg) via intraperitoneal injection and perfused transcardially with 0.9% saline (50 ml), followed by 4% paraformaldehyde fixative (50 ml) in phosphate buffer (PB; 0.1M; pH = 7.4). Brains were extracted from the calvaria, postfixed for 24 h in the same fixative, and cryoprotected in 30% sucrose in PB solution for 24 h at 4°C. Each brain was sectioned in the coronal plane at 40 µm thickness, on a sliding freezing microtome into six series and stored at 0 °C in a cryoprotectant solution (30 % ethylene glycol, 30 % glycerol, in 0.1 M PB) prior to immunohistochemical staining.

Immunohistochemistry

To assess hippocampal neurogenesis, tissue was immunolabeled for doublecortin (DCX), a microtubule-associated phosphoprotein that serves as a marker for immature neurons. Briefly, one series of free-floating sections were rinsed in PB, washed in Tris-buffered saline (TBS; pH = 7.4), incubated in TBS containing sodium meta-periodate (0.1 M; 20 min), rinsed for 30 minutes in a solution containing TBS and Triton X-100 (0.25 %; TBST) and then blocked in TBST with 3 % horse serum for 1 h. Sections were incubated with a goat anti-DCX antibody (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA) in TBST containing 1% horse serum over-night at room temperature. After several washes in TBS containing 1% horse serum, sections were incubated with secondary antibody (1:200; horse anti-goat IgG) in TBS with 1% horse serum at room temperature for 1 h. Sections were washed with TBS and incubated with avidin-biotin complex (1:500; “Elite Kit,” Vector Labs). Tissue was then washed in sodium acetate trihydrate (0.2 M) and imidazole (1.0 M) solution (pH 7.4 with acetic acid). Reaction product was visualized using an acetate-imidazole buffer containing 0.05 % 3/3'-diaminobenzidine tetrahydrochloride (DAB; Sigma, MO) and 1.0 % freshly prepared H₂O₂. Sections were washed in acetate-imidazole buffer to terminate the immunochemical reaction, mounted onto alum-submersed slides, air dried for 24 h, dehydrated through a series of graded alcohols (70 %, 95 %, and 100 %), cleared in xylene, and cover-slipped with DPX.

Quantification of DCX-positive cells

DCX-positive cells in the dentate gyrus of the hippocampus were counted using the optical fractionator method (Mouton, 2002; Overk et al., 2009; West et al., 1991, West, 1993, 1999). Specifically, we sampled every sixth section throughout the rostrocaudal extent of the dentate gyrus, including the subgranular zone and granular cell layer. Sampling included both the dorsal and ventral blades of the dentate gyrus. Stereoinvestigator 8.21.1 software (Micro-BrightField, Cochester, VT) was used to systematically sample throughout the designated region of interest. Counts were performed at predetermined intervals ($x = 230$, $y = 230$), and a counting frame ($130 \times 90 \mu\text{m} = 11700^2 \mu\text{m}$) and superimposed on the live image of the tissue sections. The sections were analyzed using a 60×1.4 PlanApo oil-immersion objective. The thickness of each section was determined by focusing on the top of the section, zeroing the z-axis followed by focusing on the bottom of the section. The

average tissue thickness was 8.3 μm with a range of 7.6 μm – 12.1 μm . The dissector height was set at 6 μm , allowing for a 2- μm top guard zone and at least a 2- μm bottom guard zone. A total of 10–12 sections were evaluated per animal. Bright field photomicrographs were taken with the aid of a Nikon microscope.

Determination of DCX antibody tissue penetration

DCX antibody penetration throughout the depth of tissue sections was determined during the optical fractionator by visual analysis of immunolabeling throughout the z-axis and post-probe run examination of depth histograms which demonstrate marker placement in the z-axis (Kelley, et al., 2011, Overk, et al., 2009). DCX antibody penetrated the full depth of the section, thus allowing for the equal probability of counting all objects, a prerequisite for unbiased stereology.

Data Analysis

Statistical analyses were conducted using the Statistical Analysis System (Version 9.1; SAS Institute, Cary, NC). The primary dependent measure for both the hidden and visible platform tasks was the mean number of errors committed. These data were analyzed using PROC GLIMMIX, a generalized linear mixed models procedure for conducting repeated measures analyses for various probability distributions including normal data (Wolfinger and O'Connell, 1993). The models used for these analyses included the between-group fixed effects: cohort (1 or 2), genotype (Ts65Dn or 2N) and maternal diet [unsupplemented (normal choline content) or supplemented], and session-block (3 sessions/session-block) or testing session (as appropriate), as well as all relevant higher-order interactions. Random effects for mouse and block were also included.

The neurogenesis data (DCX-positive cells) were analyzed using a non-parametric Kruskal-Wallis one-way analysis of variance, due to unequal variances between groups [determined by the Levene test of homogeneity (Levene, 1960)]. The primary dependent measure was the mean number of DCX-positive cells within the dentate gyrus of the hippocampus. Lastly, Spearman's rank correlation coefficient was used to assess the correlation between mean number of errors in the hidden platform task and number of DCX-positive cells.

The significance level was 5% for primary analyses of both behavioral and neurogenesis endpoints. To control for multiple comparisons following a significant overall F-test or Kruskal-Wallis test, the subsequent pair-wise comparisons were conducted using a Bonferroni procedure. Three comparisons were of interest [(1) 2N vs. Ts56Dn (2) Ts65Dn vs. Ts65Dn Ch+, and (3) 2N vs. 2N Ch+]; thus, the criteria for significance for these tests was $(.05/3) = 0.0167$.

Results

Body Weight

Analysis of body weight at the start of testing revealed a main effect of Genotype ($F_{(1, 40)} = 13.59$, $P < 0.001$) but no effect of Diet ($F_{(1, 40)} = 0.00$, $P = 0.95$), and no interaction of Diet and Genotype ($F_{(1, 40)} = 1.05$, $P = 0.32$). The mean body weight of the 2N mice (mean =

35.19 g; S.E.M. = 0.53) was significantly greater than that of the trisomic mice (mean = 31.82 g; S.E.M. = 0.405) ($P < 0.001$). This effect of the trisomy on body weight is consistent with prior reports (Bianchi et al., 2010a; Fuchs et al., 2012; Roper et al., 2012).

Hidden platform

Analysis of mean errors per session, across the 5 session-blocks (3 sessions/block) of testing in the hidden platform task, revealed a main effect of Genotype ($F_{(1, 50.2)} = 9.05$, $P < 0.01$). Although the main effect of Diet was not significant ($F_{(1, 50.2)} = 0.89$, $P = 0.35$), a significant interaction of Diet and Genotype was found ($F_{(1, 50.2)} = 6.76$, $P = 0.01$). Pair-wise comparisons show that the unsupplemented trisomic mice committed a significantly higher number of errors than the unsupplemented 2N mice ($P = 0.0003$). MCS significantly improved performance of Ts65Dn offspring relative to their unsupplemented counterparts ($P = 0.014$). No effect of MCS was detected for the 2N mice ($P = 0.25$).

Visible platform

Analysis of mean errors per session across the six days of testing did not reveal a significant main effect of Genotype ($F_{(1, 51.89)} = 0.06$, $P = 0.81$) or Diet ($F_{(1, 52.03)} = 0.54$, $P = 0.47$) nor a significant Genotype $\times$ Diet interaction ($F_{(1, 51.89)} = 3.51$, $P = 0.07$, see Fig. 2). These results indicate that the observed group differences in performance in the hidden platform task were not due to group differences in visuomotor ability, swimming ability, or motivation to find the platform.

Neurogenesis

Using unbiased stereology, the total number of DCX-positive cells was estimated within the dentate gyrus (see Fig. 3). A significant effect of treatment group ($H_{(3)} = 29.04$, $P < 0.0001$) was found for the number of DCX-positive cells. Unsupplemented Ts65Dn mice had significantly fewer DCX-positive cells than unsupplemented 2N mice ($P = 0.0001$; Fig. 4). Importantly, the Ts65Dn Ch+ mice had significantly more DCX-positive cells than unsupplemented Ts65Dn mice ($P = 0.0002$). No effect of MCS was detected for 2N mice ($P = 0.40$).

Correlation between water maze errors and adult neurogenesis

The Spearman's rank order analysis revealed a modest but significant negative correlation between water maze errors and DCX-positive cell number ($r_s(41) = -0.470$, $P < 0.001$, see Fig 5). Thus, greater neurogenesis in the DG was associated with fewer errors in the water maze. Removal of the one animal with an extreme error score did not significantly affect this relationship ($r_s(40) = -.440$, $P = 0.0036$).

Discussion

Maternal choline supplementation and impaired spatial learning/memory in the Ts65Dn mice

Unsupplemented Ts65Dn mice were significantly impaired in their ability to learn and perform the hidden platform RAWM task, a hippocampal-dependent task (Mizumori et al.,

1999; Muller et al., 1996; O'Keefe, 1976; O'Mara, 1995; Wiener, 1996). In contrast, the trisomic mice did not differ from controls in the visible platform task, which places similar demands on motor function, visual acuity, and arousal regulation, but does not require spatial mapping. Thus, the pattern of effects in these two tasks implicates impaired spatial cognition in the Ts65Dn mice, consistent with prior reports (Belichenko et al., 2007; Bimonte-Nelson et al., 2003; Chang and Gold, 2008; Escorihuela et al., 1995; Holtzman et al., 1996; Hunter et al., 2003; Reeves et al., 1995; Sago et al., 1998). Supplementing the maternal diet with additional choline during pregnancy and lactation substantially improved the performance of the adult trisomic offspring in the hidden platform task relative to their unsupplemented counterparts, demonstrating that this early dietary intervention improves spatial cognition.

These findings represent the first demonstration that MCS improves spatial learning/memory during adulthood in a mouse model of DS and AD. MCS improves spatial learning/memory in rodent models of various conditions that produce cognitive impairment in humans, including normal age-related cognitive decline (Glenn et al., 2008; Meck et al., 2003, Meck et al., 2007), prenatal alcohol exposure (Thomas et al; 2009; Thomas et al., 2010) and seizure disorders (Holmes et al., 2002; Wong-Goodrich et al., 2011; Yang et al., 2000), indicative of a neuroprotective effect that merits further consideration for translation to human populations including DS.

The absence of a beneficial effect of MCS on spatial learning of the 2N mice contrasts with several reports that pre- and/or early postnatal choline supplementation of normal rats improves spatial cognition (Cheng et al., 2008; Glenn et al., 2007, 2008; McCann et al., 2006; Meck et al., 1988; reviewed in Meck and Williams, 2003; Wong-Goodrich et al., 2008; Zeisel, 2000) and functioning of the septo-hippocampal system (Jones et al., 1999; Li et al., 2004; Pyapali et al., 1998; Steingart et al. 1998). In the present study, the lack of benefit for the 2N mice likely is due to the task not being sufficiently demanding for them. In this regard, prior reports indicate that the spatial cognition benefits of MCS for normal rodents exist for demanding tasks, particularly those which place the greatest requirements on hippocampal function (McCann et al., 2006; Meck and Williams, 1999). For example, in one prior water maze study, a benefit of MCS was seen for normal rats when the location of the escape platform changed daily but not when it remained in the same location across sessions (Tees, 1999; Tees and Mohammadi, 1999) as in the present study. The less demanding reference memory version of this task was selected only after extensive pilot testing demonstrated that the Ts65Dn mice could not solve the former, more demanding version of the test.

One final interpretive issue pertains to the order of administering the visible and hidden platform tasks. Because the visible platform task was administered after the hidden platform task, it cannot provide a pure test of associative learning because the change in task rules also placed demands on cognitive flexibility. Nonetheless, the absence of group differences in this task provides evidence that group differences in performance in the hidden platform task are unlikely to be due to sensory, motor, or motivational differences.

Lasting effects of early choline supplementation on adult hippocampal neurogenesis

Unsupplemented Ts65Dn mice exhibited a reduced number of DCX-positive cells in the hippocampus relative to the 2N mice, indicating reduced hippocampal neurogenesis in Ts65Dn mice, consistent with prior studies (Bianchi et al., 2010a; Chakrabarti et al., 2011; Clark et al., 2006; Llorens-Martin et al., 2010). However, in several of these earlier studies, BrdU was used to detect new cells but their specificity was not verified by neuronal specific markers (Bianchi et al., 2010b; Clark et al., 2006), contrary to the present study.

Importantly, the present study also demonstrated that supplementing the maternal diet with additional choline substantially increased hippocampal neurogenesis in the adult trisomic offspring. In addition to the implications of this finding for functions dependent on adult hippocampal neurogenesis (discussed below), this finding also suggests that MCS may improve developmental neurogenesis in the trisomic mice, based on evidence that adult neurogenesis is an extension of early ontogenetic neurogenesis, relying on similar molecular machinery (reviewed in Kuhn and Blomgren, 2011). Impaired ontogenetic neurogenesis in Ts65Dn mice and DS individuals likely contributes to the hypoplasia and hypocellularity observed in various brain regions (Guidi et al., 2010; Rachidi and Lopes, 2008) and the consequent developmental delay and cognitive impairments. MCS may also reduce hypocellularity, developmental delay, and cognitive impairments in humans with DS.

Although prior work has shown that MCS increases adult hippocampal neurogenesis in normal rats (Glenn et al., 2007), this effect was not seen in the 2N mice in the present study. Several factors may account for these differences including species differences and the age of the animals. In this prior study, the rats were 8 months of age at the time of the assessment, whereas the mice in the present study were much older, averaging 15.4 months of age. It is possible that the benefit of MCS on this function in normal rodents declines with aging.

Mechanisms underlying improved neurogenesis and spatial learning/memory in Ts65Dn mice

In the present study, MCS both increased hippocampal neurogenesis and improved spatial cognition in Ts65Dn mice, effects which may be causally linked. Indeed, a significant negative correlation was observed between neurogenesis in the DG and errors in the water maze, consistent with prior studies showing that treatments which impair adult hippocampal neurogenesis also disrupt spatial cognition (Deng et al., 2009; Dupret et al., 2008; Farioli-Vecchioli et al., 2008; Garthe et al., 2009; Imayoshi et al., 2008; Jessberger et al., 2009; Snyder et al., 2005; Zhang et al., 2008). These data collectively support a causal relationship between the increased hippocampal neurogenesis in the supplemented trisomic mice and their improved spatial cognition.

It is possible that the beneficial effects of MCS on spatial cognition and neurogenesis in the Ts65Dn mice reflect the effects of this early dietary manipulation on neurotrophic factors. Notably, MCS in normal rats has been shown to increase levels of brain-derived neurotrophic factor (BDNF) (Glenn et al., 2007) and nerve growth factor (NGF) (Sandstrom et al., 2002) in the brains of the adult offspring. BDNF increases survival of newly

proliferated neurons (Lee et al., 2001; Linnarsson et al., 2000; Mattson et al., 2004) and plays an important role in spatial learning and memory (Mizuno et al., 2000). Another possibility, suggested by the effects of MCS on NGF expression in normal rats, is that MCS provided target-derived neuroprotection of Ts65Dn BFCNs, which begin to atrophy in Ts65Dn mice by 6 months of age due to impaired retrograde transport of NGF (Cooper et al., 2001; Granholm et al., 2000; Holtzman et al., 1992, 1996; Salehi et al., 2006). Notably, BFCNs projecting from the medial septum to the hippocampus modulate spatial mapping (Ikonen et al., 2002; Leutgeb et al., 1999; Okada and Okaichi, 2010) and septohippocampal cholinergic activity has been shown to facilitate neurogenesis (Mohapel et al., 2005). Parallel studies in our lab provide evidence for a reduction in neurodegeneration of BFCNs in Ts65Dn mice supplemented with choline early in life (Ash et al., 2011).

Although much remains to be learned regarding the specific mechanism(s) by which MCS exerts lasting effects on cognitive functioning and neurogenesis, both effects (as well as the previously documented effects on neurotrophins and cholinergic system structure and function), likely reflect either: (i) organizational brain changes secondary to acetylcholine's role as an ontogenetic signal (Cermak et al., 1999; Meck et al., 1989; Zeisel and Niculescu, 2006); and/or (ii) epigenetic modifications with lasting effects on gene expression, secondary to choline's role as a methyl donor (Niculescu et al., 2004, 2006; Waterland and Jirtle, 2003; Zeisel, 2009a).

Other manipulations shown to increase neurogenesis in Ts65Dn mice

Other treatments have been shown to improve neurogenesis in Ts65Dn mice, but the translational potential is much lower for these interventions than for MCS. First, the combination of environmental enrichment plus exercise has been shown to increase neurogenesis in both trisomic and 2N mice (Chakrabart et al., 2011), but these findings do not imply a therapeutic effect of this intervention because the group differences are more accurately interpreted as showing the adverse effect of environmental isolation on neurogenesis than the therapeutic effects of enrichment (discussed in Strupp and Beaudin, 2006). Another treatment that has been found to increase neurogenesis in both Ts65Dn and 2N mice is neonatal or adult administration of fluoxetine (Bianchi et al., 2010a; Clark et al., 2006). However, it is unlikely that this treatment would be advocated clinically in light of evidence that this drug increases risk of malformations and cardiovascular abnormalities when given to humans during fetal development (for review see Morrison et al., 2005), and exacerbates the behavioral deficits of Ts65Dn mice when given during adulthood (Heinen et al., 2012). A final treatment that has been shown to increase neurogenesis in Ts65Dn mice is lithium, although in this case only adult treatment has been evaluated (Bianchi et al., 2010b). Again, translational potential is limited by reports of hypothyroidism (discussed in McKnight et al., 2012) and renal toxicity (Grunfeld and Rossier, 2009) with chronic administration of the drug. Hypothyroidism would be of particular concern in the case of early developmental therapy, due to the lasting adverse effects of this condition on brain development (Auso et al., 2004; Williams and Hume, 2008).

Conclusions and Clinical Implications

Growing evidence indicates that not only is increased maternal intake of choline safe for both mother and developing fetus, but that it may be necessary for optimal brain development and lifelong cognitive and affective functioning of the offspring. As noted above, lasting beneficial effects of this maternal intervention have been reported for offspring spatial cognition (Cheng et al., 2008; Glenn et al., 2007; Meck et al., 1988; Meck and Williams, 1999; Wong-Goodrich et al., 2008; Zeisel, 2000), attentional function (Mohler et al., 2001; Moon et al., 2010; Powers et al., 2011), and emotion regulation (Cheng et al., 2008; Moon et al., 2010), as well as protection against age-related cognitive decline (McCann et al., 2006; Meck et al., 1988; 2007). These beneficial effects of increased maternal choline intake likely reflect the intensified demand for choline during fetal development (Jiang et al., 2012; Yan et al., 2012), coupled with the apparent inadequacy of standard rodent chow to provide sufficient choline to meet these needs. This latter inference is based on the evidence that pregnancy causes a pronounced depletion of choline pools in rats consuming standard laboratory chow (Holmes-McNary et al., 1996; McMahon and Farrell, 1985). The increased demand for choline during fetal development likely reflects the numerous ontogenetic roles of this nutrient, including serving as a precursor for membrane phospholipids and the neurotransmitter, acetylcholine, as well as serving as the primary source of methyl groups for methylation reactions, including DNA and histone methylation, which play important roles in regulating gene expression (discussed in Zeisel 2009a, b).

Although the heightened demand for choline during pregnancy is reflected in a slight increase in choline intake recommendations for pregnant women relative to non-pregnant women, the increment is small (425 vs. 450 mg/choline/day) (IOM, 1998), and viewed by many as inadequate to meet the demands of pregnancy and lactation (Craciunescu et al., 2003; Zeisel, 1995; Zeisel, 2000, 2009b; Zeisel and da Costa, 2009). The current recommended intake level for adults (including pregnant women), determined only recently in 1998, was based on the quantity of choline required to prevent liver dysfunction; brain function did not factor into this recommendation. Indeed, in light of the growing evidence from maternal choline supplementation studies (reviewed in Meck and Williams, 2003) as well as data demonstrating the increased choline demands of pregnancy (e.g., Holmes-McNary et al., 1996; Jiang et al., 2012; McMahon and Farrell, 1985; Yan et al., 2012), many researchers have called for a re-evaluation of choline intake recommendations for pregnant women (e.g., Jiang et al., 2012; Meck and Williams, 1999, 2003; Yan et al., 2012; Zeisel, 1995, 2009b).

In sum, the present study demonstrated that supplementing the maternal diet with additional choline during pregnancy and lactation improves spatial cognition and hippocampal neurogenesis in adult Ts65Dn offspring. If these findings generalize to humans, MCS could provide a therapy to normalize brain development and cognitive function in DS as well as possibly slow the neurodegeneration associated with both DS and AD (Ash et al., 2011). Moreover, because the animal literature indicates beneficial effects for both normal offspring as well as those with DS, this type of nutritional advice could be given to all women and thereby circumvent the problem that such treatments need to be implemented during the earliest stages of development to have the greatest impact, and yet prenatal testing

is not performed in the majority of pregnancies yielding DS offspring (discussed in Newberger, 2000). Future studies are needed to ascertain whether the beneficial effects of MCS are seen in humans.

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ABBREVIATIONS

BFCNs	Basal forebrain cholinergic neurons
BDNF	Brain-derived neurotropic factor
DAB	3/3'-diaminobenzidine tetrahydrochloride
DG	Dentate gyrus
2N	Disomic mice born to dams on a normal choline diet
2N Ch+	Disomic mice born to dams on a diet supplemented with additional choline
DCX	Doublecortin
DS	Down syndrome
HSA21	Human chromosome 21
MCS	Maternal choline supplementation
MMU16	Mouse chromosome 16
NGF	Nerve growth factor
PB	Phosphate buffer
PND	Postnatal day
TBST	TBS with Triton X-100
TBS	Tris-buffered saline
Ts65Dn	Ts65Dn mice born to dams on a normal choline diet
Ts65Dn Ch +	Ts65Dn mice born to dams on a diet supplemented with additional choline

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Highlights

- We test a novel therapy for Down syndrome (DS), using the Ts65Dn mouse model.
- Maternal choline supplementation improved spatial cognition in Ts65Dn offspring.
- Maternal choline supplementation increased hippocampal neurogenesis in Ts65Dn mice.
- Spatial learning ability was correlated with adult hippocampal neurogenesis.
- Increased maternal choline intake may reduce cognitive dysfunction in humans with DS.

Figure 1.

Mean ($\pm$ SE) errors in the Hidden Platform task, (A) plotted as a function of session block (3 sessions per block) and (B) averaged across the 15 sessions: MCS improved performance of the adult Ts65Dn offspring in the Hidden Platform task of the radial arm water maze, a hippocampal-dependent task. * $p < 0.01$.

Figure 2. Mean (+/- SE) errors in the Visible Platform task across the 6 sessions of testing. The groups did not differ in their performance in the Visible Platform task.

Figure 3. DCX-positive cells in the subgranular zone and granular cell layer of the hippocampus across different treatment groups. Adult hippocampal neurogenesis is deficient in Ts65Dn mice (C, D) compared to 2N littermates (A, B). Choline supplementation partially normalized adult neurogenesis in Ts65Dn mice (G, H). No effect of maternal choline supplementation was observed in 2N mice (E, F). h = hilus.

Figure 4.

Mean (+/- SE) number of DCX-positive cells in the dentate gyrus of the hippocampus. Unsupplemented Ts65Dn mice expressed significantly fewer DCX- positive cells than the 2N mice ($P < 0.0001$). Maternal choline supplementation significantly increased the number of DCX positive cells for the Ts65Dn mice ($P < 0.001$). * $p < 0.001$.

Figure 5.

Correlation between mean errors in the water maze and DCX-positive cell number, showing the line of best fit. A negative correlation was seen between mean errors in the water maze and DCX-positive cell number in the dentate gyrus ($r_s(41) = -.470$, $P < 0.001$); i.e. as hippocampal neurogenesis increased, the number of errors decreased.