BENEFITS OF MATERNAL CHOLINE SUPPLEMENTATION IN A MOUSE MODEL OF DOWN SYNDROME: ELUCIDATION OF UNDERLYING NEURAL MECHANISMS

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

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August 2014

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BENEFITS OF MATERNAL CHOLINE SUPPLEMENTATION IN A MOUSE MODEL OF DOWN SYNDROME: ELUCADATION OF UNDERLYING NEURAL MECHANISMS

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Down syndrome (DS) is the most common known cause of intellectual disability (ID), affecting 1 in 800-1000 births. This disorder is caused by triplication of human chromosome 21(HSA21) due to nondisjunction during meiosis. In addition to ID, individuals with 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). Although there are currently no effective treatments for either the ID or dementia seen in this disorder, it is hoped that the recent development of murine models of this disease will aid in elucidating the pathogenic mechanisms and testing potential therapies. The Ts65Dn mouse model of DS survives to adulthood and exhibits key features of this disorder, notably impairments in learning, attention, and memory, as well as deficient ontogenetic neurogenesis and degeneration of basal forebrain cholinergic neurons (BFCNs) in the medial septum (MS). The overarching goal of the present thesis was to use this mouse model to study the role of cholinergic atrophy in the age-related cognitive decline seen in DS (Chapter 3), and to test the effectiveness of one promising therapy for DS (Chapter 1 and 2): maternal choline supplementation (MCS).

Our studies revealed that Ts65Dn mice exhibit attentional dysfunction as early as 3 months of age, which becomes more pronounced with aging. Moreover, assessment of cholinergic neurons in the nucleus basalias of meynert/substantia innominata revealed a reduced density of these neurons in both young and old trisomic mice (relative to 2N), but no age-related

changes in the count, density or size of these neurons was observed with aging as is commonly believed. Our subsequent studies using MCS revealed that the Ts65Dn offspring of dams supplemented with additional choline during pregnancy and lactation exhibited improvements in spatial cognition, relative to Ts65Dn offspring of dams on the control diet. In addition, MCS significantly increased adult neurogenesis of the trisomic offspring and offered protection to BFCNs in the MS. These results provide exciting new evidence that MCS may represent a safe and effective treatment approach for expectant mothers carrying a DS fetus, as well as a possible means of BFCN neuroprotection during aging for the population at large.

BIOGRAPHICAL SKETCH

Ramon Velazquez Jr. received his B.A. degree from the Department of Psychology at California State University, Long Beach in May of 2007. He then worked as a project director at the Greater Los Angeles Veteran affairs Nicotine research unit in 2008 where he studied treatment options for smoking cessation within the veteran population. He received his Ph.D. degree in 2014 from Cornell University. His research focuses on preventive therapies for the cognitive deficits in both developmental and neurodegenerative disorders such as Down syndrome and Alzheimer's disease. He has so far coauthored more than 4 publications in peer-reviewed journals in addition to 2 first author papers in high impact peer-reviewed journals. He has had continuous funding for his research since his undergraduate days, via a National Institute of Mental Health undergraduate grant, in addition to a pre-doctoral grant from the National Science Foundation. He has received a variety of awards for his work on the benefits of maternal choline supplementation in the Ts65Dn mouse model of Down syndrome: for example he was the recipient of the 2013 James Bradford Neurobehavioral Teratology Society award. Dr. Velazquez will be joining the laboratory of Dr. Oddo Salvatore at the Sun Health Research Institute in Sun city, AZ in September 2014 where he plans on investigating therapies for the cognitive deficits seen in Alzheimer's disease.

ACKNOWLEDGEMENTS

This dissertation would not have been possible without the guidance and help of several individual who in one way or another contributed and extended their valuable assistance in the preparation and completion of the studies presented next. First and foremost, I would like to thank Dr. Barbara J. Strupp, Professor in the Nutritional Science department at Cornell University, for her help and guidance. Dr. Strupp has been my inspiration as I hurled through the obstacles of completing these studies. I would also like to thank Dr. Elliot J. Mufson, Professor in the department of Neurological sciences at Rush University, and his graduate student Christy Kelley, for their help with training and processing of tissue samples utilized in our studies. I would also like to thank Dr. Jessica Ash and Dr. Brian Powers, both members of the Strupp lab at the time the studies were ongoing, for their help with data collection and animal care. I would also like to thank Dr. Stephen D. Ginsberg, Associate professor at the Nathan Klein Institute in New York, for his training on brain removal techniques. I would also like to Myla S. Strawderman for her help and guidance with running the statistical analyses. I would also like to thank my committee members, Dr. David M. Smith, Dr. David L. Deitcher, Dr. Vivian Zayas, and Dr. Harry G. Segal for their help and guidance throughout my academic career. Last but not least, I would like to thank my family and friends for their continued care and support throughout my graduate career.

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ABBREVIATIONS

Basal forebrain cholinergic neurons (BFCN)

beta-amyloid plaques (Aβ)

Brain-derived neurotropic factor (BDNF)

3/3′-diaminobenzidine tetrahydrochloride (DAB)

Choline acetyltransferase (ChAT)

Dentate gyrus (DG)

Disomic mice born to dams on a normal choline diet (2N)

Disomic mice born to dams on a diet supplemented with additional choline (2N Ch+)

Disomic mice that started testing at 3 months of age (2N-Y)

Disomic mice that started testing at 12 months of age (2N-O)

Doublecortin (DCX)

Down syndrome (DS)

Down Syndrome critical region (DSCR)

Human chromosome 21(HSA21)

Hippocampus (HP)

Inter trial interval (ITI)

Maternal choline supplementation (MCS)

Mouse chromosome 16 (MMU16)

Medial septum (MS)

NAPVSIPQ peptide (NAP)

Nucleus basalis of meynert/ substantia innominata (NBM/SI)

Nerve growth factor (NGF)

pan-neurotrophin receptor ($p75^{NTR}$)

Phosphate buffer (PB)

Postnatal day (PND)

Quantitative polymerase chain reaction (qPCR)

Radial arm water maze (RAWM)

S-adenoyslmethionine (SAM)

SALLRSIPA peptide (SAL)

TBS with Triton X-100 (TBST)

Tris-buffered saline (TBS)

tyrosine receptor kinase A (TrkA)

Ts65Dn mice born to dams on a normal choline diet (Ts65Dn)

Ts65Dn mice born to dams on a diet supplemented with additional choline (Ts65Dn Ch +)

Ts65Dn mice that started testing at 3 months of age (Ts65Dn-Y)

Ts65Dn mice that started testing at 12 months of age (Ts65Dn-O)

Ventral diagonal band (VDB)

Visible platform task (VP)

INTRODUCTION

Down Syndrome in Humans

Affecting 1 in every 800-1,000 live births and currently seen in over 5 million globally (Roizen and Patterson, 2003), Down syndrome (DS) is the most common known cause leading to intellectual disabilities (ID). The primary predisposing factor for DS is advanced maternal age (Abruzzo and Hassold, 1995). DS is caused by a sporadic event known as nondisjunction during meiosis that leads to an extra copy of all or part of the long arm of human chromosome 21 (HSA21; Rachidi and Lopes, 2008). The disease is characterized by numerous somatic abnormalities such as heart and gastrointestinal defects (van Trotsenburg et al., 2006), immunological (Cruz et al., 2009) and pulmonary diseases (Cooney and Thurlbeck, 1982; Schloo et al., 1991), and epilepsy (Roizen and Patterson, 2003; Sorge and Sorge, 2010). ID is amongst the most severe problems affecting individuals with DS (Noble, 1998; Rachidi and Lopes, 2008; Roizen and Patterson, 2003). During fetal development and at birth, a variety of neuropathological deficiencies (e.g., hypocellularity, deficient neurogenesis, apoptosis) are evident in regions such as the cerebellum (a structure important in proprioceptive-motor control and motor learning) and the hippocampus (a structure important in spatial and dissociative learning and memory; Contestabile et al., 2007; Guidi et al., 2011). Furthermore, individuals with DS develop age-related cognitive decline due to the onset of Alzheimer's disease (AD) -like neuropathology at the third-fourth decade of life (Isacson et al., 2002; Sendera et al., 2000; Whitehouse et al., 1982, Wisniewski et al., 1985a). Researchers believe that this may due to the Amyloid precursor protein (APP) gene, a culprit in AD, being located on HSA21 (Wilcock and Griffin, 2013). Currently, there are no approved therapies for the ID and cognitive decline during mid-life seen in humans with DS.

Brain pathology in DS without AD

Numereous reports have found that the brains of DS patients are smaller in size compared with normal individuals, suggesting a casual link between ID and brain hypothrophy (i.e., decrease volume and degeneration; Aylward et al., 1997; Aylward et al., 1999; de la Monte and Hedley-Whyte, 1990; Jernigan et al., 1993; Pinter et al., 2001; Rotmensch et al., 1997; Schmidt-Sidor et al., 1990; Sylvester, 1983; Teipel et al., 2003; Winter et al., 2000; Wisniewski, 1990). In fetuses and children with DS, post-mortem tissue studies have revealed brain hypotrophy that is associated with hypocellularity and cortical dysplasia (Golden and Hyman, 1994; Ross et al., 1984; Wisniewski, 1990). Studies measuring neurogenesis (i.e., the production of new neurons) in DS post-mortem fetal brain tissue have detected reductions in the number of newly born cells that is believe to contribute to the observed brain hypotrophy (Contestabile et al., 2007). Specifically, the cerebellum, a structure that is important in the regulation of proprioceptive-motor control and motor learning, demonstrates growth disturbances during the second-trimester that is solely due to reductions in neurogenesis (Guidi et al., 2008; 2011). The hippocampus, a structure that is important in assocaitve learning and memory as well as spatial cognition, also shows reductions in neurogenesis during fetal development (Contestabile et al., 2007). However, unlike the cerebellum, the hippocampus also shows increased apoptosis and a higher number of cells going to a glial phenotype and fewer cells expressing neuronal markers were evident (Guidi et al., 2011). This suggests that there are different factors contributing to the decreased volume within certain brain structures in DS.

Data from both post-mortem tissue and neuroimaging studies in DS patients have revealed that changes beginning early in life become more prominent in adolescence and throughout adulthood (reviewed in Nadel, 2003). Magnetic resonance imaging (MRI) volumetric anal-

yses have detected reductions in brain volume, including reduced gray and white matter in addition to regional differences in areas such as the cerebellum, frontal and temporal lobes of children (Kates et al., 2002; Pinter et al., 2001) and young adults (Nadel, 2003; Menghini et al., 2011). Smigielska-Kuzia and colleagues (2011) found that the volume of both the left and right hippocampus is significantly reduced in 8 year-old DS patients. This is further confirmed in adults with DS who have not reached the AD dementia. Weis (1991) applied both stereological techniques and MRI to post-mortem tissue samples of DS patients with ages ranging from 30-45 years and found that the whole brain was significantly smaller. Specific brain regions were also smaller in volume in this study, including the cortex and cerebellum. Collectively, these findings suggest that the brains of DS patients continue to show deficiencies from childhood throughout adulthood that is likely the basis of ID throughout life.

Behavioral deficits in DS

Assessing learning and memory abilities in infants has proven difficult due to structures such as the hippocampus not becoming fully functional until many months after birth; perhaps even as long as 16-18 months of age (Nadel and Wilner, 1989; Nadel and Zola-Morgan, 1984). One study examining basic learning in 3 month old infants with DS found that they performed normally, in terms of initial learning, acquisition, speed and retention, in a task that required learning about contingencies between their own movements (e.g. kicking their leg) and reinforcement (Ohr and Fagen, 1993). At 9 months however, this same group found that DS infants were impaired in learning about the contingency between arm movements and reinforcement (Ohr and Fagen, 1994). This may be due to controls performing better, thus making the deficit appear more profound in DS infants. These researchers concluded that there is a relative decline

in conditionability in infants with DS after 6 months of age. These researchers suggested that this might coincide with the increasing brain abnormalities after 6 months of age.

Mangan (1992) was one of the first researchers to examine hippocampal-dependent deficits in toddlers with DS. Mangan (1992) tested toddlers who were 26-30 months of age since it is known that place learning does not emerge until around 18 months, which coincides with estimates on when the hippocampus itself becomes functional (Magan and Nadel, 1990). Mangan (1992) found that toddlers with DS do in fact show deficits in place learning, which requires the use of cues in the surrounding environments to guide the subject to a hidden object and is dependent on the hippocampus. DS infants were not impaired in a cued-learning task that requires subjects to locate one visible object; this latter finding is hippocampal-independent (Mangan, 1992). The deficits in place learning may be related to the hypocellularity and deficient neurogenesis seen within the hippocampus of DS fetuses and children (Contestaible et al., 2007; Guidi et al., 2008).

Language development is another area of dysfunction in DS. We will only briefly discuss deficits in language development in infants and children with DS, since it is not a focus of this dissertation.

Most infants with DS are delayed at saying their first words (i.e., show a longer delay from babbling to speech), their vocabulary grows more slowly than in ordinary children and although they use the same range of two- word phrases as all children, they have difficulty in mastering the many rules for talking in grammatically correct sentences (Chapman, 1997; reviewed in Chapman and Hesketh, 2000; Stoel-Gammon. 2001). As infants with DS progress into childhood and early adulthood, they are restricted to short telegraphic utterances (keywords without the function words, for example "went swimming Dad" rather than "I went swimming last night

with my Dad") and also tend to have difficulty in pronouncing words clearly (Bray and Woolnough, 1988). Researchers suggest that language and speech is important for other cognitive abilities. For example, the storage and recall of information from long term memory is dependent on organizing the information on the basis of meanings conveyed by language. Thus, any serious language delay will inevitably result in increasing cognitive delay (Chapman and Hesketh, 2000; Chapman, 1997). This is further supported by work showing that children with DS show deficits in verbal short-term working memory (Chapman and Hesketh, 2000; Constanzo et al., 2013). Collectively, these results show that language development is delayed in DS patients and that these delays may lead to deficits in cognitive functioning.

Executive functions are a set of high cognitive abilities that control and regulate other functions and behaviors (Welsh et al., 1991). These processes include short term and working memory, reasoning, inhibition, behavioral flexibility, planning and problem solving (Costanzo et al., 2013; Friedman et al., 2006; Pennington and Ozonoff, 1996). Studies have shown that patients with DS are impaired in certain components of executive functions and this impairment becomes progressively worse as patients enter early onset AD dementia (Rowe et al., 2006).

Constanzo and colleagues (2013) more recently investigated executive functions in DS patients ranging from ages 8.6 to 21.2 years (who did not show signs of dementia) and found impairments in an auditory-sustained attention task (Manly et al., 1999, 2001), which requires subjects to count silently tones and announce the total number of these tones at the end of each trial. Visual-selective attention, measured by the Sky search task (Manly et al., 1999, 2001) that requires subjects to circle pairs of identical artifacts as quickly as possibly, was also impaired in DS patients relative to mental age-matched controls. Further impairments were seen in the forward digit span task (Orsini et al., 1987), a test of verbal short-term memory, which requires

subjects to recall a sequence of digits that was read to them. Verbal working memory, which is also measured using the digit span task but requires subjects to recall the digits in the reverse order, was also impaired in DS patients. Visual-spatial short-term and working memory were also impaired in DS patients as evident by impaired performance in the Corsi block test (Orsini et al., 1987), which requires subjects to reproduce a sequence of blocks in either the same order (short-term memory) or in the reverse order (working memory). Lastly, response inhibition, as measured by the stroop task was impaired in these DS children. Collectively, these findings suggest that executive functions are impaired in young individuals with DS that may affect adaptive behaviors.

One last areas that is of particular importance to discuss in this introduction, as it is a primary component in the chapters ahead, is that of spatial learning and memory, which is mediated in part by the hippocampus and becomes noticeably impaired in DS individuals during adolescence and adulthood (Caltagirone et al., 1990; Devenny et al., 1992; Ellis et al., 1989; Pennington et al., 2003). Pennington and colleagues (2003) examined hippocampal function in DS children ranging from 12 – 16 years of age. They administered a spatial long-term memory task (Thomas et al., 2001) that is adapted from the water maze task used in animal models of learning and memory and requires animals to use contextual cues in the environment to remember the location of a submerged platform. Utilizing a spatial map requires intact spatial memory skills; animals with lesions to the hippocampus are generally not able to use spatial cues to create this kind of spatial map. In the human version, subjects use a joystick to move around a computer-generated virtual arena. During the first four trials, the children had to locate a blue rug on the floor of the arena. After the fourth trials, children were to locate the blue rug by using contextual cues, although they were not instructed to use cues. Whenever the subjects located the area

where the blue rug was previously, a sound came on letting them know they were in the right location. After five trials, a probe trial was given where children were not notified that they had found the location of the rug. If the subjects had utilized the contextual cues in the virtual environment to develop a spatial map of the arena, he or she should have spent the majority of the probe trial searching in the correct quadrant. Results showed that DS children spent less time searching in the correct quadrant then controls during the probe trial, suggesting that they were unable to form a spatial map of the environment, suggesting hippocampal dysfunction. Several other studies have found that adults with DS show marked deficits in long-term memory (Caltagirone et al., 1990; Devenny et al., 1992; Ellis et al., 1989), mediated by the hippocampus. An example of these impairments comes from a study that used a book of pictures in which subjects had to both recognize specific items and also remember there location; DS adults were impaired in the recall task when long delays were implemented, suggesting long-term memory deficits.

In sum, decades of reports have shown that individuals with DS are impaired in a range of cognitive functions starting from infancy up to adulthood. Researchers suggest that these impairments are likely due the brain pathologies evident in fetuses and children with DS that continue throughout adulthood (Contestabile et al., 2007; Guidi et al., 2008, 2011; Aylward et al., 1997; Aylward et al., 1999; de la Monte and Hedley-Whyte, 1990; Jernigan et al., 1993; Pinter et al., 2001; Rotmensch et al., 1997; Schmidt-Sidor et al., 1990; Sylvester, 1983; Teipel et al., 2003; Winter et al., 2000; Wisniewski, 1990). Further compromising cognitive functions in DS patients is the start of AD-like neuropathology that is imminent in all patients with DS (Isacson et al., 2002; Sendera et al., 2000; Whitehouse et al., 1982, Wisniewski et al., 1985a).

Alzheimer's disease (AD) in DS patients

Greater than 98% of DS patients develop AD-like neuropathology at around the third-fourth decade of life, which is characterized by beta-amyloid (Aβ) plaques, neurofibrillary tangles and degeneration of BFCN neurons (Lott and Dierssen, 2010; Ness et al., 2012; Wilcock, 2012; Zigman and Lott, 2007). This strong increase in AD-like neuropathology during midlife has been attributed to the triplication of the amyloid precursor protein APP gene on HSA21, which yields higher levels of Aβ, the main constituent of plaques in AD that are toxic to nerve cells in the brain (Ness et al., 2012; Wilcock and Griffin, 2013). This is further supported by work showing that an elderly adult with DS who had a microdeletion resulting in APP disomy did not develop dementia or classic AD neuropathology (Zigman and Lott, 2007). The location and progression of Aβ plaques and neurofibrillary tangles in patients with DS at autopsy mimic those found in the general AD population (reviewed in Ness et al., 2012). Both Aβ plaques and neurofibrillary tangles are thought to further contribute to the cognitive deficits in DS patients.

One of the most prominent neuropathological changes in both DS and AD, which is particularly important to discuss, as it is the basis of chapter 2 and 3 in this dissertation, is that of degenerating BFCN neurons (Bowen et al., 1976; Casanova et al., 1985; Davies and Maloney, 1976; Whitehouse et al., 1982; Yates et al., 1980). Cholinergic neurons within the basal forebrain project to the entire cortical mantle and hippocampus (Mesulam et al., 1983) and are important in modulating functions of these target regions. The basal forebrain is divided into the medial septum (MS), diagonal band, and nucleus basalis of meynert (NBM) and together form a single cholinergic system whose three regions differ principally in their projections (Mesulam et al., 1983; Casanova et al., 1985). The NBM sends projections to the neocortex and is important in modulating attention (Gratwicke et al., 2013). The MS sends cholinergic projections to the hippocam-

pus and is important in modulating functions such as spatial and dissociative memory (reviewed in Everitt and Robbins, 1997). Reductions in the size and number of cholinergic neurons within the NBM and MS have been observed in postmortem tissue of DS patients ages >59 years (Casanova et al., 1985). Although the exact cause of BFCN atrophy in humans with DS has not been identified, animal models (see mouse model section) have shed light on the possible mechanism leading to such pathologies, which will be discussed in a proceeding section.

Documenting the cognitive deficits after the onset of AD in DS patients has proven somewhat difficult because of the preexisting ID. There is evidence however that the nature of these deficits tends to mimic those seen in general AD (Oliver et al., 1998). Deterioration in learning, memory and orientation are typically the first signs/symptoms of AD in DS patients (Cosgrave et al., 2000). Further impairments in executive functioning have been shown when comparing DS patients with and without AD (Rowe et al., 2006). For example, there are slight declines in verbal and long-term memory for those over 50 years of age with DS, and the ability to form long-term memories and visual spatial construction may be impaired (see Stanton and Coetzee, 2004). Demented DS patients experience cognitive deficits such as forgetfulness of recent events, geographically orientation, loss of previously learned skills and confusion. There is also evidence of personality changes, social isolation, increase dependence, and excessive uncooperativeness. Selective attention, which is the ability to focus on a predetermined stimulus while ignoring irrelevant or distracting stimuli, is also deficient in DS patients with AD pathology (Krinsky-Mchale, et al., 2008). Collectivity, these data show that DS patients exhibit further deficits in cognitive functions with the onset of AD-like neuropathology during midlife.

Experimental treatments utilized in humans with DS and AD

There are currently no clinically approved treatments for the ID or dementia observed in DS patients. Several therapeutic interventions have been utilized to try and ameliorate the cognitive dysfunctions seen in DS. This section will focus on therapies aimed at improving the cognitive dysfunction of DS patients with and without AD.

The first set of drugs that have been tested in an effort to ameliorate the cognitive deficits in DS target specific neurotransmitter systems. Donepezil, which is a cholinesterase inhibitor and blocks the breakdown of acetylcholine, only showed improvements in language (Heller et al., 2003, 2004; Johnson et al., 2003; Kishnani et al., 2009) in children with DS. Other cognitive dysfunctions were not ameliorated with this drug in both children and adults with and without AD (see de la Torre and Dierssen, 2012). These findings in DS adults with AD are of particular importance since BFCN atrophy is evident in these patients, suggesting that simply increasing acetylcholine may not be sufficient to ameliorate the cognitive deficits. Rivastigmine, an inhibitor of acetylcholineesterase similar to Donepezil, also failed to improve language and cognition in both children and adults with DS (Heller et al., 2010; Prasher et al., 2005). One drug that has not yet been tested in DS patients that holds promise is Galantamine. Galantamine is a reversible and competitive cholinesterase inhibitor and is also an allocentric ligand, thus increasing acetylcholine binding at nicotinic receptors. It is used in AD and has had success in improving memory deficits. Researchers suggest that it may be the next drug utilized to treat and ameliorate the cognitive deficits that arise with AD pathologies in DS (de la Torre and Dierssen, 2012).

Acetyl-carnitine, derived from carnitine, has been reported to display beneficial properties, such as increasing activity of cholinergic neurons, membrane stabilization, and mitochondrial antioxidant activity in AD patients without DS (Palacios et al., 2011). This compound was

administered to 40 individuals with DS for a 6-month period, but failed to show any improvements in cognitive functions (Pueschel, 2006). Nicotine patches have also been tested in DS patients, specifically because of the cholinergic deficits with the onset of AD. The testing of such treatment has been done in a very limited number of DS patients, however. Interestingly, results have shown improvements in attention and information processing; researchers suggest a more thorough investigation to confirm these benefits (Bernert et al., 2001; Seidl et al., 2000).

Memantine is another treatment option that gained attention due to its benefits in the Ts65Dn mouse model of DS (Rueda et al., 2010). Memantine is an uncompetitive NMDA receptor antagonist that was approved as a treatment option in severe AD (Mohan et al., 2009). The rationale for its use in DS is that it should help reduce abnormal activation of excitatory glutamatergic neurotransmission. Abnormal excitatory glutamatergic neurotransmiion leads to seizures and disruptions in memory formation (reviewed in Francis, 2005). In the general AD population, this drug has already showed benefits in cognitive functioning and slowing of the decline seen in AD overtime (Mohan et al., 2009). Currently, there is an ongoing clinical trial in young adults with DS that is examining improvements in memory and other cognitive abilities. In sum, memantine helps those with pathologies relevant to AD in the general population, making it a possible therapeutic intervention for DS patients with AD.

The treatments presented above constitute the main clinical trials performed in DS subjects, none of which have shown compelling results (reviewed in de la Torre and Dierssen, 2012). Not having the ability to investigate the neural level changes within the brains of DS patients upon testing these treatments makes it difficult to troubleshoot and understand why they may or may not ameliorate cognitive deficits. The introduction of the Ts65Dn mouse model of DS by Davisson (1990) has led to a more complex understanding of DS both at the behavioral

and neural level. This has consecutively led to the development of treatments that one day may prove beneficial to those with DS.

The Ts65Dn mouse model of DS

In an effort to further understand the disease process seen in DS and consequently develop treatments that may help ameliorate deficits, a mouse model that mimics the human disease was developed (Davisson, et al., 1990). The Ts65Dn mouse was the first segmental trisomy created and is in now the best characterized and most-widely used 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 100 genes orthologous to those on HSA21 (Mural et al., 2002; Patterson and Costa, 2005). This triplicated chromosomal segment also includes the “Down syndrome critical region,” (DSCR) which is considered necessary, although not solely sufficient, for the DS phenotype (Belichenko et al., 2009; Olson et al., 2007, 2004). 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). The most important feature of this mouse model is that it exhibits central nervous system deficits similar to humans with DS. Notably, this mouse model shows deficiencies in developmental and adult neurogenesis (Bartesaghi et al., 2011; Bianchi 2010a, b.; Chakrabarti et al., 2011; Clark et al., 2006; Llorens-Martin et al., 2010; Velazquez et al., 2013) and AD-like neuropathology during midlife (Cooper et al., 2001; Granholm et al., 2002; Holtzman et al., 1996). In addition, the mouse model exhibits impairments in hippocampal-mediated learning and memory (reviewed in Crnic and Pennington, 2000; Davison and Costa, 1999; Patterson et al., 2005; Reeves et al., 1995), motor

dysfunction (Costa et al., 1999), delays in achieving sensorimotor milestones (Holtzman et al., 1996), lack of behavioral inhibition (Coussons-Read and Crnic, 1996), attentional dysfunction and deficits in emotional regulation (Driscoll et al., 2004; Moon et al., 2010), and deficiencies in operant learning (Wenger et al., 2004) when the demands of the task increase.

Like humans with DS (discussed above; Contestaible et al., 2007, Guidi et al., 2008, 2011; Rachidi and Lopes, 2008), Ts65Dn mice exhibit widespread neurogenesis reduction during fetal brain development that may lead to the numerous neurological deficits (Bianchi et al., 2010a, 2014). Neurogenesis reductions during pre- and early post-natal life have been observed in brain regions such as the subgranular zone of the dentate gyrus (DG), subvenrticular zone of the lateral ventricles, striatum, neocortex and cerebellum (Bartesaghi et al., 2011; Bianchi et al., 2010). This is consistent with the post-mortem reports in fetuses with DS that show deficiencies of neurogenesis in the hippocampus (Guidi et al., 2008). This suggests that reductions in neurogenesis during development may underlie the reductions in brain size (Aylward et al., 1997; Aylward et al., 1999; de la Monte and Hedley-Whyte, 1990; Jernigan et al., 1993; Pinter et al., 2001; Rotmensch et al., 1997; Schmidt-Sidor et al., 1990; Sylvester, 1983; Teipel et al., 2003; Winter et al., 2000; Wisniewski, 1990) in humans with DS and furthermore may be associated with the cognitive impairment seen in both humans and the Ts65Dn mouse model of DS.

Evidence for a continued deficiency in adult neurogenesis has also been demonstrated in the Ts65Dn mouse model. The proliferation and survival of new neurons throughout adulthood in mammalian species is confined to 2-brain regions. This includes the hippocampus and subventricular zone of the lateral ventricles (reviewed in Braun and Jessberger, 2014). Ts65Dn mice show deficient adult neurogenesis in both these brain regions, which likely contributes to dysfunctions 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) and olfactory learning (Bianchi et al., 2014). Interestingly, Mohapel and colleagues (2005) found that adult hippocampal neurogenesis is modulated by cholinergic activity from the MS. Thus one possibility for continued deficiencies in adult hippocamal seen in adult Ts65Dn mice may be due to the degeneration of MS neurons, which fail to modulate hippocampal function.

AD-like neuropathology in Ts65Dn mice

At around 4-6 months of age, Ts65Dn mice begin to show a progressive loss of basal forebrain cholinergic neurons (BFCN; Granholm et al., 2002; Holtzman et al., 1996). The basal forebrain provides the major cholinergic inputs to the neocortex (e.g., attention; Driscoll et al., 2004; Moon et al., 2010) and hippocampus (e.g., explicit memory function; Hyde et al., 2001; Hyde and Crnic, 2001), thus modulating functions associated with these target regions. The atrophy of BFCNs has been attributed to a malfunction in the trafficking of neurotrophins, specifically the neurotrophin “nerve growth factor” (NGF). Cooper and colleagues (2001) found that the retrograde transport of NGF was faulty in 6 months old TS65Dn mice that carry a triplication of the APP gene. Importantly, they found that if you infuse NGF directly into the cell body, neurons that were beginning to show signs of atrophy would develop a healthier phenotype. This suggests that the atrophy of BFCNs in the Ts65Dn mice is due to a malfunction in NGF transport from the terminal button to the synapse and not a lack of its availability. In sum, the Ts65Dn mouse model of DS shows behavioral and neural deficits that mimic the human disease and furthermore make it a suitable model to test potential therapeutics.

Treatments utilized in the Ts65Dn mouse to ameliorate cognitive deficits

A series of pharmacotherapies have been tested in the Ts65Dn mouse and have shown significant amelioration of cognitive dysfunction. Most of the pharmacotherapies designed to improve cognitive functions have been attempted in the mouse model during the adult stages (reviewed in Bartesaghi et al., 2011). There have however been recent studies that have found benefits of treatments administered prenatally (Guidi et al., 2014, Incerti et al., 2012; Moon et al., 2010; Velazquez et al., 2013) and since prenatal testing can identity triplications of chromosomal abnormalities in humans, this allows the opportunity of early interventions that may help improve brain development in fetuses with DS. Here we will discuss treatments utilized in the Ts65Dn mouse during early development, adulthood and during fetal development.

Both fluoxetine and lithium have been tested in Ts65Dn mice in an effort to increase neurogenesis, serotonin and cognitive functions. Both these drugs affect the serotonergic system by increasing the amount of serotonin in the synaptic cleft (Wang et al., 2008). Studies have shown that both humans with DS and this mouse model show reduced levels of serotonin (Whitaker-Azmitia, 2001). Furthermore, serotonin has been linked to developmental neurogenesis (Faber and Haring, 1999; Whitaker-Azmitia, 2001), thus decreases in serotonin may account for deficiencies of neurogenesis in the mouse model.

Bianchi and colleagues (2010a) administered Fluoxetine to neonatal Ts65Dn mice for 13 days (PND 3-PND15) and measured neurogenesis at both PND 15 and PND 45. Fluoxetine did in fact normalize the number of adult born neurons at both time points in the hippocampus and subventricular zone of Ts65Dn neonates. A subset of fluoxetine treated trisomics were tested on a hippocampal-dependent fear conditioning task and were found to perform significantly better than those trisomics that did not receive fluoxetine. Guidi and colleagues (2013) took a subset of

these same animals and found that the granule cells from the hippocampi of Ts65Dn mice had a severely hypotrophic dendritic arbor, fewer spines and a reduced innervation then 2N mice. Fluoxetine corrected such deficiencies and furthermore, they found that serotonin levels, which were deficient in Ts65Dn mice, were normalized by the fluoxetine. Even more recently, Guidi and colleagues (2014) administered fluoxetine to Ts65Dn pregnant dams from embryonic day 10 until birth. The brains of these offspring were extracted either 2hrs or 45 days after birth. Ts65Dn mice offspring that were not treated with fluoxetine were found to have severe neuro-genesis reductions and hypocellularity throughout the forebrain, mid brain and hindbrain, in-cluding the hippocampus at both time points. Ts65Dn mice treated with prenatal fluoxetine showed full restoration of neurogenesis in all brain regions at both time points. In addition, fluoxetine treated Ts65Dn mice performed significantly better on a hippocampal-dependent fear-conditioning task. Although these findings seem encouraging and researchers have recom-mended fluoxetine as a treatment option in humans with DS, one study actually found behavioral impairments in >7month old T65Dn mice that received fluoxetine for 4 weeks (Heinen et al., 2012). Even more concerning was an observed increase in seizure activity and mortality in fluoxetine treated Ts6Dn mice. One possibility for such discrepancies may be the age of admin-istration, as Bianchi (2010a) and Guidi's (2013, 2014) studies examined fluoxetine early in de-velopment while Heinen (2012) examined administration later in adulthood. It's possible that at later ages, fluoxetine may do more harm then provide benefits. Another cautionary note comes from studies revealing that human mothers taking fluoxetine while pregnant can have newborns with malformations, cardiovascular abnormalities (for review see Morrison et al., 2005), and even spontaneous abortions (Nakhai-Pour et al., 2010). In sum, it appears that fluoxetine admin-

istration requires further examination in the mouse model before being suggested as a potential therapy for humans with DS.

Bianchi and colleagues (2010b) found that 1-month of lithium administration, in diet, was sufficient to correct deficient neurogenesis in the subventricular zone of 12-month Ts65Dn mice. A more recent study by this same group found that adding lithium to the diet of 5-6 month old Ts65Dn mice for 1-month was sufficient to increase the proliferation of neuronal precursors and restore adult-hippocampal neurogenesis in the DG (Contestaible et al., 2013). These animals also performed significantly better in several behavioral tasks, including fear conditioning, object location, and novel object recognition task. The findings from these two studies suggest that lithium may be a suitable treatment option for correcting deficiencies in adult neurogenesis and ameliorating cognitive deficits in DS.

Melatonin is another compound that was administered to middle aged (5-6 month old) Ts65Dn mice for 5 months in an effort to ameliorate cognitive deficits as well as protect from degeneration of BFCNs (Corrales et al., 2013). Melatonin plays key roles in circadian rhythms, sleep homeostasis, behavioral modulation, and aging (Hardeland et al., 2011) and decreases with age, which is thought to contribute to age-related neurodegeneration (Corrales et al., 2013; Reiter et al., 1998). Treatment in AD patients has been shown to reduce Aβ neurotoxicity, apoptosis, neuroinflammation, oxidative stress, and degeneration in BFCNs (Dragicevic et al., 2011; Feng et al., 2004; Matsubara et al., 2003; Olcese et al., 2009). Administration for 5 months in Ts65Dn mice improved spatial learning and memory and increased the number of ChAT-positive cells within the MS. These results suggest that melatonin may be a suitable treatment option for the protection of BFCNs and spatial learning and memory.

The use of treatments during fetal development provides a unique opportunity to attempt

to correct early deficiencies in neurogenesis that are thought to cause widespread hypocellularity in DS (Guidi et al., 2010). Only a few studies have attempted such treatments in the TS65Dn mouse model of DS. As previously discussed, fluoxetine from embryonic day 10 to birth led to benefits in neurogenesis and hippocampal dependent tasks (Guidi et al., 2014). Although it could be suggested that fluoxetine corrected the deficits in neurogenesis, it is hard to say whether these benefits would have held up through adulthood. Without examining the offspring at later ages, it is hard to say whether such benefits are long lasting.

A few studies have examined the lasting benefits of treatments administered during fetal development. Incerti and colleagues (2012) administered daily 20μg injections of vasoactive intestinal peptide, NAPVSIPQ (NAP) and SALLRSIPA (SAL), to pregnant Ts65Dn mice from embryonic day 8 to 12. These two peptides have revealed therapeutic effects for developmental delay and learning deficits in various diseases. Furthermore, theses peptides have been shown to increase the survival of DS cortical neurons (Busciglio et al., 2007). Ts65Dn offsrping were tested at 8-10 months of age on a hippocampal-dependent Morris water maze task. The findings revealed that Ts65Dn offspring of dams treated with the NAP + SAL compound learned the location of the hidden platform significantly faster. These researchers suggest that this early intervention may have corrected the deficient neurogenesis and hippocampal hypocellularity, but no neural data were presented in this paper. Although this treatment seems as a possibility for DS, further work is required to confirm whether this early intervention corrected neuropathologies.

Our laboratory previously reported that supplementing the maternal diet of Ts65Dn dams with addtitional choline (CH) provides lasting benefits for the trisomic offspring in a task of focused and sustained attention (Moon et al., 2010). This has led to further questions of whether MCS may be a suitable treatment option for other cognitive abilities and whether neuropatholo-

gies may be reduced in the mouse model. A detailed description of maternal CH as a potential treatment option for DS is presented next.

Maternal choline supplementation as a potential treatment for DS

Growing evidence suggests that the current recommended daily intake of choline for pregnant women may not be optimal for fetal development and lifelong health of the offspring. Two decades of research with normal rodents has shown that increasing maternal intake of choline (4.5X the amount in normal chow) produces lasting improvements in offspring cognitive functioning (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). More recent rodent studies have shown that increasing maternal choline intake also increases offspring neurogenesis (Glenn et al., 2007), cholinergic activity (Meck et al., 1989, 2007), and brain neurotrophin levels (Glenn et al., 2007; Sandstrom et al., 2002). Furthermore, a study in our laboratory found that supplementing the maternal diet with additional choline significantly improved attentional functioning in the Ts65Dn mouse model of Down syndrome (Moon et al., 2010). Collectively, these studies suggest that current estimates of adequate choline intake may not be sufficient during pregnancy to allow optimal fetal development and offspring cognitive functioning. Below, I provide background on choline and possible mechanisms for these beneficial effects, which is central to the studies in this dissertation.

What is choline?

Choline is an essential nutrient, grouped with the vitamin B complex, which plays key

roles in fetal development. During development, CH is a source of major dietary source of methyl groups for the production of S-adenosylmethionine (SAM), which is a substrate for epigenetic mechanisms, such as DNA methylation, that can alter gene expression throughout life (Niculescu et al., 2002). Choline is also a precursor of the neurotransmitter acetylcholine (Blusztajn and Wurtman, 1983), which is essential for proper organization and function of the developing brain through its effects on neurogenesis and synaptic formation (reviewed in Jiang et al., 2014; Meck and Williams, 2003). Furthermore, CH is important in the metabolism of fats (Vance, 1990), and is a precursor of phosphatidylcholine, which is used in the synthesis of the constructional components in the bodies' cell membranes (Hanada et al., 1991). The important role of CH during fetal development may explain why insufficient amounts (insufficient relative to needs) may result in suboptimal fetal development.

CH can be found in a variety of foods. Foods highest in total CH concentrations per 100g include: beef liver (418 *mg*), chicken liver (290 *mg*), eggs (251 *mg*), wheat germ (152 mg), bacon (125 *mg*), dried soybeans (116 *mg*), and pork (103 *mg*). Researchers suggest that CH-rich foods are an important component of the diet and that especially during pregnancy it would be prudent to include them as part of a healthy diet in order to ensure adequate fetal development.

CH during pregnancy and lactation

As evident by the major roles that CH plays during fetal development (see section above), CH is in high demand during pregnancy and lactation. Studies have shown that there is a pronounced depletion of CH-derived methyl groups in pregnant vs non-pregnant women (Jiang et al., 2014; McMahon and Farrell, 1985; Sweiry et al., 1986; Sweiry and Yudilevich, 1985). Moreover, the plasma and serum CH concentrations are 6 or 7-fold higher in the fetus and neonate than they

are in the mother (Zeisel et al., 1980; Zeisel and Wurtman, 1981), suggesting a transfer from the mother to the fetus. In pregnant rats, total liver CH stores are diminished compared to nonpregnant controls (Zeisel et al., 1995), suggesting that endogenous pools of CH are also utilized due to the demands imposed by the developing fetus. Furthermore, because milk contains a great deal of CH, lactation further increases the demand maternal CH, resulting in further depletion of tissue stores (McMahon and Farrell, 1985; Holmes-McNary et al., 1996). These observations suggest that the demand for choline rises dramatically during pregnancy and that choline intake must be increased to promote optimal fetal development.

Studies measuring CH levels in pregnant women have found that CH is positively associated with cognitive measures in the offspring both early after birth and later on in childhood. For example, Wu and colleagues (2012) found that maternal plasma concentrations of choline and betaine, the metabolite of choline, at 12 weeks of gestation were positively associated with measures of early cognitive development in the offspring, indicating a possible benefit of CH supply on cognitive function in humans. Similarity, Boeke and colleagues (2013) reported that children born to mothers in the highest quartile of choline intake performed significantly better on a measure of visual memory at 7 years of age. In sum, these findings suggest that CH is very important during the gestation and lactation and is associated with cognitive functions after birth.

Supplementing the maternal diet with CH during pregnancy

Although these epidemiological data are provocative, it is possible that they reflect confounding with other nutrients. For that reason, stronger evidence is provided by studies that have experimentally increased choline intake during pregnancy and/or lactation. Numerous studies have demonstrated that the offspring of dams supplemented with additional choline during preg-

nancy and/or lactation (about 4.5 X the amount in control chow) performed significantly better on tests of spatial memory than the offspring of dams on a control diet meeting the RDI for choline (Meck et al., 1988; Meck et al., 1989; Meck and Williams, 1997a; Meck and Williams, 1999; Williams et al., 1998). Similar performance increases have been reported for prenatal CH supplemented rats on temporal processing tasks that require divided attention or increments in attention following a change in the predictiveness of a stimulus (Lamoureux et al., 2008; Meck and Williams, 1997a). In sum, these studies suggest that offspring of CH supplemented dams are characterized in adulthood by improved performance relative to control rats in tasks measuring spatial memory, temporal processing, and attention (Brandner, 2002; Meck et al., 1988, 1989; Meck and Williams, 1997a; Ricceri and Berger-Sweeney, 1998; Zeisel et al., 1991). Supplementing the maternal diet with additional CH has also been shown to have lasting facilitative effects on offspring cholinergic activity (Meck et al., 1989, 2007), adult neurogenesis (Glenn et al., 2007), and brain levels of neurotrophins (*NGF* and *BDNF*) in rodents (Glenn et al., 2007; Sandstrom et al., 2002). Although much remains to be learned concerning the mechanisms by which these lifelong effects are produced, it has been hypothesized that they reflect lasting changes in gene expression, secondary to epigenetic mechanisms, due to choline's role as the major dietary source of methyl groups (Zeisel, 2004).

These studies have shown that there are two critical periods when MCS produces most pronounced affects on health outcomes, memory function and longevity (McCann et al., 2006; Meck and Williams, 2003). Meck and colleagues (2007) reported that these two critical periods include gestational days 12–17 and postnatal days 16–30 in rodents. In light of these findings, recent studies suggest supplementing with CH during the entire gestation and lactation (i.e. perinatal) due to the high need in the developing fetus (Holmes-McNary et al., 1996; McMahon and

Farrell, 1985; Zeisel et al., 1980; Zeisel and Wurtman, 1981).

Although the majority of research on optimal maternal CH has been conducted in rodent models, the effects of additional maternal CH supplementation has been recently examined in humans. In a randomized placebo-controlled study, Ross and colleagues (2013) evaluated the effect of supplementing pregnant women with 900mg CH/day, which is twice the current RDI by IOM, from the second trimester through birth. Newborns were then supplemented with an additional 100mg CH/day (vs placebo) up to the third month after birth. The results showed that CH supplementation (vs placebo) yielded more infants within the normal cerebral inhibition range at the 5th postnatal week, indicating that the additional CH facilitates appropriate sensory gating development (Ross et al., 2013). Although much more remains to ascertain the exact benefits of additional CH during fetal development, the current findings in both rodents and humans suggest that the current RDI may not be optimal for proper development and the RDI may need reconsideration.

These observed lasting facilitative effects of maternal choline supplementation seen in normal rats and humans have triggered numerous studies looking at the potential neuroprotective effects in various types of disease processes. Recently, it has been shown that perinatal (gestation and lactation) CH supplementation improves cognitive functioning in a mouse model of Down syndrome (Ts65Dn) when tested as adults (Moon et al., 2010). Perinatal CH supplementation also exerts lasting neuroprotective effects, as evidenced by a reduction in MK-801-induced neurotoxicity (Guo-Ross et al., 2002) as well as attenuation of memory impairment associated with senescence (Blusztajn, 1998; Meck and Williams, 1997b; Meck and Williams, 2003), epileptic-seizures (Yang et al., 2000) and prenatal alcohol exposure (Thomas et al., 2000). Collectively, these studies indicate that availability of CH during early development has significant

neuronal organizational effects and may increase the restorative and/or neuroprotective capacity of the adult brain.

A possible concern is the potential dangers, if any, of consuming too much CH during pregnancy. Researchers have shown that in order to experience side effects from high doses of CH (e.g. low blood pressure, fishy odor smell, and diarrhea), an 8-9 fold increase of the DRI needs to be consumed (about 3.5 - 4 grams; WebMD, 2009). This in unlikely due to the current suboptimal consumption in humans (Fischer et al., 2007) and furthermore, studies on the benefits of addition maternal CH in rodents has suggest an increase of no more then ~4.5 X the RDI to see benefits (e.g. Albright et al. 1999a, Albright et al., 199b; Meck and Williams, 1988; Moon et al., 2010); one study found benefits in humans with only 2 X the RDI amount (Ross et al., 2013), which is nowhere near the 8-9 fold increase that shows adverse effects. Overall, these results suggest that supplementing with additional CH during pregnancy and lactation is safe and should pose no side effects when given 2 – 4.5 X the RDI amount.

The current recommended daily intake amount and the need for reconsideration

The findings in both normal rodents and humans, as well as various models of neurological disorders suggest that the current recommended intake amount for preganant mothers may not be optimal for fetal develoopment and lifelong cognitive functioning of the offspring. The current established recommedations for CH intake for pregnant mothers were established in 1998 by the IOM based on the estimated level of CH intake required to prevent liver damage (Institute of Medicine, 1998). The current established adequate intake level for CH is 550 *mg/day* for men aged 19 years and older, 425 *mg/day* for women aged 19 and older, 450*mg/day* for pregnant women, 550*mg/day* for lactating women, and 18 *mg/kg /day* for infants

(Institute of Medicine, 1998). A study examining daily human CH intake on an *ad libitum* diet found averages to be lower then the RDI, especially in women (Fischer et al., 2007). In the same study, 6 out of 26 men developed CH deficiency symptoms while consuming the adequate intake, suggesting that the current DRI may not be sufficient. Furthermore, Shaw et al. (2004) found that 25% of pregnant women from a California study showed intakes of less than half the DRI. These findings suggest that 1) the current RDI amounts may not be optimal and 2) that pregnant may not even be consuming the current RDI, perhaps leading to deficiencies and further compromising fetal development.

To date, the available data suggest that pregnant and lactating women are the most sensitive to availability of dietary CH because of the greatly increased need for CH to form the fetus and produce milk (Zeisel et al., 1980; Zeisel et al., 1981; Zeisel et al., 1995). Researchers believe that adjusting for additional CH intake during gestation and lactation will allow for proper fetal development, which includes adequate building of cell membranes, proper precursor to build acetylcholine, which leads to proper neurogenesis and building of synapses, adequate availability of methyl groups for regulation of gene expressions (Zeisel, 2006).

The following chapters

This dissertation encompasses three chapters, each describing a single study. The first two chapters describe studies designed to determine whether supplementing the maternal diet with additional CH improves spatial cognition in the Ts65D mouse model of DS and assess two possible underlying neural mechanisms for the improved cognitive functioning seen in this model following MCS: (1) increased adult hippocampal neurogenesis and (2) protection of cholinergic neurons within the basal forebrain, which normally atrophy starting at 6 months of age, due

to the onset of AD. The third chapter was designed to characterize NBM/SI cholinergic neurons in young and aged Ts65Dn mice, describing the changes that occur with aging and investigate whether these changes correspond to progressive attentional dysfunction in these animals as they age.

CHAPTER 1

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

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

INTRODUCTION

Down syndrome (DS) is the most common known cause of intellectual disability, affecting 1 in 800-1000 births. This disorder is caused by triplication of human chromosome 21(HSA21) due to nondisjunction 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 100 highly conserved genes that are orthologous to those on HSA21 (Mural et al., 2002; Patterson and Costa, 2005; Sturgeon and Gardiner, 2011). This triplicated chromosomal segment also includes the "Down syndrome critical region," (DSCR) which is considered necessary, although not solely sufficient, for the DS phenotype (Belichenko et al., 2009; Olson et al., 2007, 2004).

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., 2001; Hyde and Crnic, 2001). 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 (Granholm et al., 2000; Holtzman et al., 1992; Holtzman et al., 1996; Hyde and Crnic, 2001) 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 × 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 X 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, 2001). 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 H_2O_2. 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 x 90 μm = 11700^2 μm) and superimposed on the live image of the tissue sections. The sections were analyzed using a 60 x 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 task 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

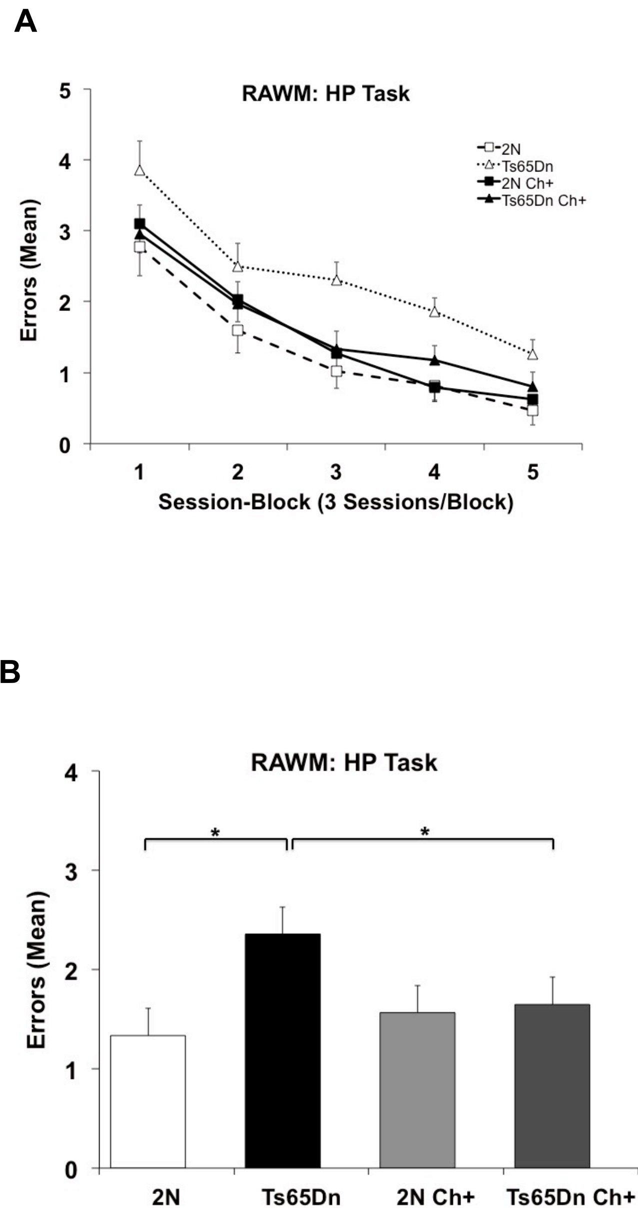

Figure 1. Mean (+/- SE) errors in the Hidden Platform task. (A) Plotted as a function of session block (3 sessions per block) and **(B)** averaged across the 15 sessions: MCS improved performance of the adult Ts65Dn offspring in the Hidden Platform task of the radial arm water maze, a hippocampal-dependent task. * $p \leq 0.01$.

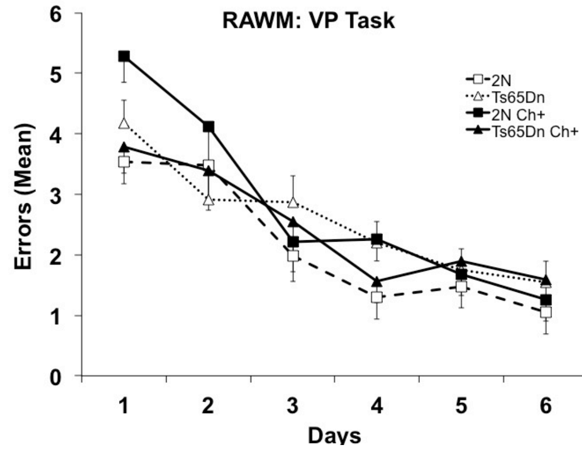

Figure 2. Mean (+/- SE) errors in the Visible Platform task across the 6 sessions of testing. The groups did not differ in their performance in the Visible Platform task.

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 × 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 correla

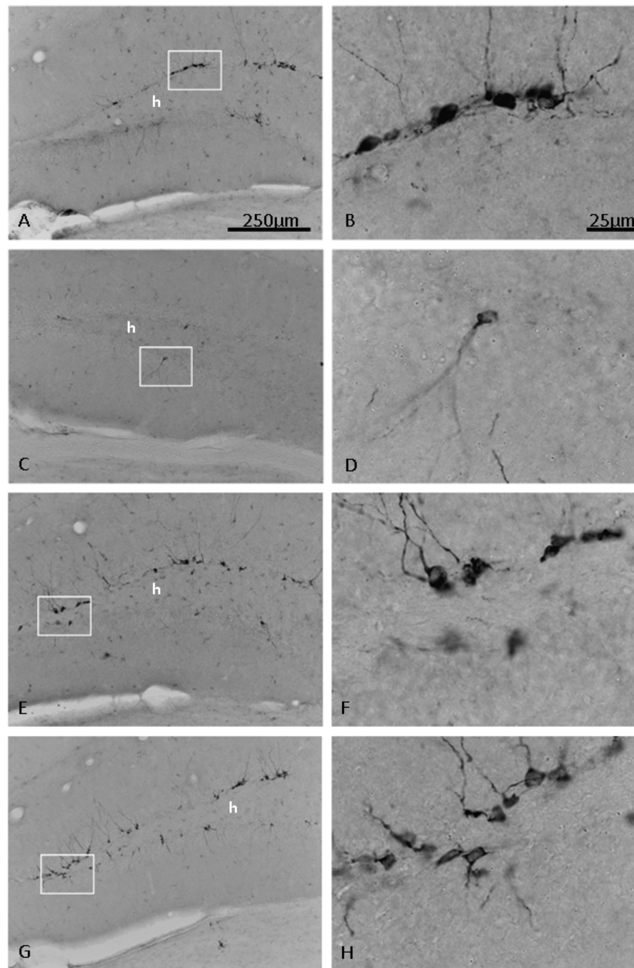

Figure 3. DCX-positive cells in the subgranular zone and granular cell layer of the hippocampus across different treatment groups. Adult hippocampal neurogenesis is deficient in Ts65Dn mice **(C, D)** compared to 2N littermates **(A, B)**. Choline supplementation partially normalized adult neurogenesis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

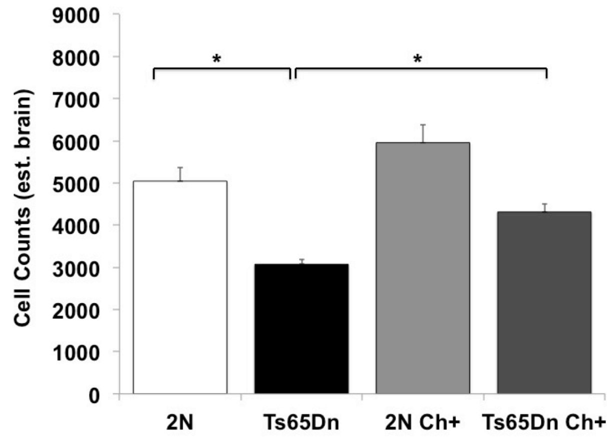

Figure 4. Mean (+/- SE) number of DCX-positive cells in the dentate gyrus of the hippocampus. Unsupplemented Ts65Dn mice expressed significantly fewer DCX- positive cells than the 2N mice ($P < 0.0001$). Maternal choline supplementation significantly increased the number of DCX positive cells for the Ts65Dn mice ($P < 0.001$). * $p < 0.001$.

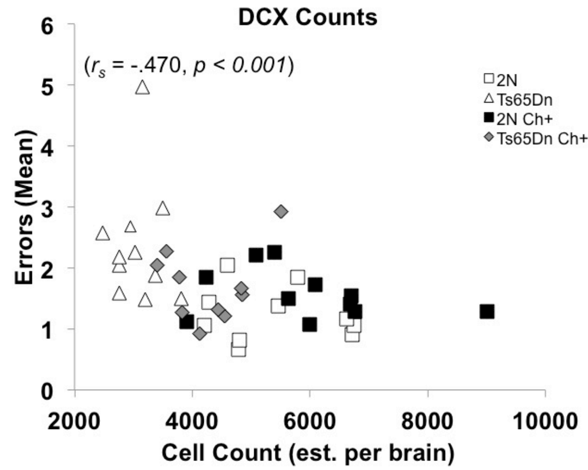

Figure 5. Correlation between mean errors in the water maze and DCX-positive cell number, showing the line of best fit. A negative correlation was seen between mean errors in the water maze and DCX-positive cell number in the dentate gyrus ($r_s(41) = -.470$, $P < 0.001$); i.e. as hippocampal neurogenesis increased , the number of errors decreased .

tion 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., 2011; 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 functional 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 at 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 ef-

fects 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.

CHAPTER 2

Maternal choline supplementation improves spatial mapping and increases basal forebrain cholinergic neuron number and size in aged Ts65Dn mice

ABSTRACT

Down syndrome (DS) is marked by intellectual disability (ID) and early-onset of Alzheimer's disease (AD) neuropathology, including basal forebrain cholinergic neuron (BFCN) degeneration. The present study tested the hypothesis that maternal choline supplementation (MCS) lessens hippocampal dysfunction and protects against BFCN degeneration in the Ts65Dn mouse model of DS and AD. During pregnancy and lactation, dams were assigned to either a choline sufficient (1.1 $^{g}/_{kg}$ choline chloride) or choline supplemented (5.1 $^{g}/_{kg}$ choline chloride) diet. Between 13 and 17 months of age, offspring were tested in the radial arm water maze (RAWM) to examine spatial learning and memory followed by unbiased quantitative morphometry of BFCNs. Spatial mapping was significantly impaired in unsupplemented Ts65Dn mice relative to normal disomic (2N) littermates. Additionally, a significantly lower number and density of medial septum (MS) hippocampal projection BFCNs was also found in unsupplemented Ts65Dn mice. Notably, MCS significantly improved spatial mapping and increased number, density, and size of MS BFCNs in Ts65Dn offspring. Moreover, the density and number of MS BFCNs correlated significantly with spatial memory proficiency, providing powerful support for a functional relationship between these behavioral and morphometric effects of MCS for the trisomic offspring. Thus, increasing maternal choline intake during pregnancy may represent a safe and effective treatment approach for expectant mothers carrying a DS fetus, as well as a possible means of BFCN neuroprotection during aging for the population at large.

INTRODUCTION

Down syndrome (DS), caused by triplication of human chromosome 21 (HSA21), is the most common genetic disorder resulting in intellectual disability (ID). By the third or fourth decade of life, DS individuals develop dementia and the histopathological characteristics of Alzheimer's disease (AD) (Casanova et al., 1985; Fodale et al., 2006; Mann, 1988; Mann et al., 1986, 1984; Mann and Esiri, 1989; Wisniewski et al., 1985a, 1985b), including neurofibrillary tangles, neuritic plaques, and cholinergic basal forebrain pathology (Mufson et al., 2003; Isacson et al., 2002; Sendera et al., 2000; Whitehouse et al., 1982). Currently, there are no therapeutic interventions that prevent or reverse ID, age-related cognitive impairment, or brain pathology in DS.

Several mouse models have been developed to study the relationship between the triplication of specific genes in DS and distinct phenotypic features (see Das and Reeves, 2011; Rueda et al., 2011; Salehi et al., 2006 for reviews). The most well-characterized animal model is the Ts65Dn mouse, which is segmentally trisomic for the distal region of mouse chromosome 16 (MMU16) which contains more than 100 highly conserved genes that are orthologous to those on HSA21 (Sturgeon and Gardiner, 2011). This triplicated chromosomal segment also includes the "Down syndrome critical region," (DSCR) which is considered necessary, although not solely sufficient, for the DS phenotype (Belichenko et al., 2009; Olson et al., 2007, 2004).

Similar to humans with DS, Ts65Dn mice are born with an intact basal forebrain cholinergic neuron (BFCN) system, which undergoes progressive atrophy starting between four and six months of age (Granholm et al., 2000; Holtzman et al., 1996; Hunter et al., 2003; Seo and Isacson, 2005). BFCN atrophy coincides with a decline in hippocampal-dependent memory function in Ts65Dn mice likely due to impairment of the cholinergic hippocampal projection system aris-

ing from the medial septum (MS) and vertical limb of the diagonal band (VDB) (Bimonte-Nelson et al., 2003; Crnic and Pennington, 2000; Fernandez and Garner, 2008; Granholm et al., 2000; Holtzman et al., 1996; Hunter et al., 2003; Hyde and Crnic, 2001; Hyde et al., 2001; Rye et al., 1984; Stasko and Costa, 2004). Cholinergic neurons within the nucleus basalis of Meynert (NBM) also atrophy, which correlates with cognitive decline in both DS and AD (Bierer et al., 1995; Davis et al., 1999; DeKosky et al., 2002; Mufson et al., 2003).

Based on these findings, interventions that maintain BFCN viability are likely to improve cognition in DS. A therapy which may hold promise in this regard is supplementation of the maternal diet with additional choline, a hypothesis based on two converging lines of evidence: (a) the pattern of cognitive impairment in DS (and the Ts65Dn mouse) is indicative of dysfunction of BFCNs and their projections to the hippocampus and frontal cortex (spatial memory and attention, respectively); and (b) in normal rodents, supplementing the maternal diet with additional choline (~ 4.5X the amount in normal chow) enhances these two cognitive domains in the offspring, and exerts structural and functional changes in the septo-hippocampal cholinergic system (reviewed in Meck and Williams, 2003; McCann et al., 2006; Zeisel and Niculescu, 2006). Prior findings from our lab have provided support for this hypothesis, demonstrating that maternal choline supplementation (MCS) produces lifelong improvements in performance of Ts65Dn offspring in behavioral tasks dependent on BFCN projections to the frontal cortex (Moon et al., 2010), and normalizes hippocampal neurogenesis in these animals (Velazquez et al., 2013).

The current study was designed to extend this prior work by testing the hypothesis that MCS improves performance of aged Ts65Dn offspring on tests of spatial learning and memory, and that the mechanism(s) underlying these cognitive benefits are related to protection of the BFCN hippocampal and cortical projection systems. To examine these hypotheses, we assessed

the effects of supplementing the maternal diet with additional choline (versus a control diet with standard choline content) in Ts65Dn offspring and their normal disomic (2N) littermates, with respect to (1) radial arm water maze (RAWM) performance, and (2) morphometric indices of MS, VDB, and nucleus basalis of Meynert/ substantia innominata (NBM/SI) cholinergic neurons. The morphology of cholinergic neurons was characterized by using 3 cholinergic markers, (1) choline acetyltransferase (ChAT), the enzyme which synthesis choline, (2) the pan-neurotrophin receptor $p75^{NTR}$, which is a low affinity neurotrophin receptor and (3) the nerve growth factor (NGF) receptor tyrosine receptor kinase A (TrkA), which is a high affinity neurotrophin receptor. Staining for ChAT allows for the characterization of all cholinergic neurons whereas labeling for $p75^{NTR}$ and TrkA is thought to provide for a better index of functioning cholinergic neurons as these receptors are lost during the degenerative process seen with the onset of AD.

METHODS

Subjects

Breeder pairs (Ts65Dn female and C57Bl/6J Eicher × C3H/HeSnJ F1 male mice) were purchased from Jackson Laboratories (Bar Harbor, ME), mated at Cornell University, (Ithaca, NY), and randomly assigned to either a choline-controlled, standard rodent chow diet (AIN-76A with 1.1 g/kg choline chloride; Dyets Inc., Bethlehem, PA) or a rodent chow diet with choline supplementation (AIN-76A with 5.0 g/kg choline chloride; Dyets Inc) as reported previously (Kelley et al., 2014a, 2014b; Velazquez et al., 2013). The two levels of maternal choline intake selected for these studies with the Ts65Dn model were based on numerous prior studies demonstrating lasting cognitive benefits of increased maternal choline intake in normal rodents (Meck and Williams, 1999; 2003; Meck et al., 2007). The choline content of the control diet is consid-

ered to provide adequate choline intake during pregnancy (Meck et al., 2007). The choline-supplemented diet provided approximately 4.5 times the concentration of choline in the normal diet, within the range of dietary variation observed in the human population (Detopoulou et al., 2008). The breeder pairs were provided *ad libitum* access to their assigned diet. Pups were weaned on postnatal day 21 (PND 21) and given *ad libitum* access to the control diet.

Breeder pairs yield litters with segmentally trisomic (Ts65Dn) mice and disomic (2N) littermates. At weaning, tail snips were sent to Jackson laboratories (Bar Harbor, ME) for genotyping by quantitative polymerase chain reaction (qPCR) for the detection of the extra chromosomal segment of MMU16, and determination of $Pde6b^{rd1}$ homozygosity. $Pde6b^{rd1}$ is a recessive mutation that leads to retinal degeneration (Keeler, 1966); mice homozygous for the $Pde6b^{rd1}$ mutation were excluded from the study.

After weaning, the pups were group-housed with same-sex littermates. Only male mice were used for these experiments. To prevent obesity (which can interfere with water maze performance), a daily ration was calculated to yield body weights that were approximately 90% of their free-feeding weights. Two weeks prior to behavioral testing, mice were singly housed to prevent fighting between cage-mates, which often occurs when group-housed male mice of this strain are returned to the home-cage following daily behavioral testing. A combination of daily handling, testing and providing objects in the home-cage (i.e., plastic igloos, tubes, and plastic-gel bones, Nestlets) countered the environmental impoverishment of single animal housing. Mice were housed in a room with a 12:12-hour reversed light-dark cycle (lights off at 8:00 a.m.) and tested during the dark cycle. The behavioral testing room directly adjoined the housing room, preventing any light exposure during the transport of the animals between rooms.

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: Radial arm water maze (RAWM)

The RAWM was used to assess spatial learning and memory. Variations of this task have been used successfully in prior studies with Ts65Dn mice (Bimonte-Nelson et al., 2003; Howell and Gottschall, 2012; Hunter et al., 2003; Lockrow et al., 2011; Velazquez et al., 2013) and other AD mouse models (Arendash et al., 2004). Because this task requires mice to navigate the arms of the maze to find the escape platform, it prevents the thigmotactic behavior exhibited by Ts65Dn mice in the Morris water maze.

The RAWM was configured in a pool (100 cm diameter) containing 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. Water temperature was maintained at 20–22 ˚C, cool enough to ensure adequate motivation to perform the task, but sufficiently warm to avoid hypothermia. The escape platform was a cylinder (surface 10 cm diameter, 7.5 cm tall) made of clear plastic, tinted black, situated 1 cm below the water surface. Because the inside of the pool was also black, the escape platform was not visible to the animals. Numerous distinctive extra-maze cues were available to allow the mice to navigate using spatial mapping. Two individuals conducted the behavioral testing, each testing an equal number of mice per treatment group. Animals were tested in two consecutive cohorts, in a stratified randomized balanced design across 4 groups,

resulting in an age range of 12.8 to 16.7 months (Mean = 15.5, SD = 1.75; see Table 1) at the start of testing. Behavioral testing followed a 20-day protocol after which the animals were immediately sacrificed at ages 13.7 – 17.8 months. Whenever possible, one Ts65Dn and one 2N male pup were selected from each litter for behavioral testing. Experimenters conducting the behavioral testing were blind to the subject's genotype and maternal diet.

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

Training

For one session prior to maze testing, the mice were acclimated to the maze and the basic procedures of the task (e.g., swimming, finding the hidden platform). During this phase, 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 the first day of the hidden platform task (see below) but on all subsequent trials, all arms were accessible.

Hidden platform (HP) task

The HP task began on the day following training. In this task, mice were tested for five trials per session for a total of 15 sessions. The escape platform remained in the same location throughout testing for each animal, with the animals starting from a different arm on each of the five 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 platform; if the platform was not located within that period, the mouse was guided to the platform. If at least half of the ani-

mal's body entered a non-goal arm an error was tallied. After each trial, each animal was given a 15 second rest period on the platform and then returned to its home cage during the inter-trial interval (ITI) in order to prevent hypothermia, a particular concern for Ts65Dn mice (Iivonen et al., 2003; Stasko and Costa, 2004; Stasko et al., 2006). All mice were tested on trial 1 before any mouse was tested on trial 2, etc., with the result that each animal had a 10–20 minute rest period between consecutive trials. The animals were weighed every other day and fed immediately following the last trial of each day.

Visible platform (VP) task

On the day following completion of the HP task, the mice were tested on the VP task for five sessions, using the same water maze apparatus. Successful performance in the VP task does not require spatial mapping but depends on other abilities needed for success in the HP task, including the ability to swim, and climb onto the platform, motivation to escape from the water, arousal regulation, and visual acuity. In the VP task, a black curtain was placed around the water maze to obscure extramaze cues. The location of the platform was indicated by a prominent intramaze cue: a tall, white PVC pipe affixed to the platform (3.8 cm diameter X 30.5 cm high). The location of the platform was changed on every trial. All other testing characteristics were the same as in the HP task, including the amount of time allotted per trial to search for the platform, the resting period on the platform, and the ITI.

Tissue preparation

Upon completion of behavioral testing, mice were deeply anesthetized with ketamine (85 mg/kg)/xylazine (13 mg/kg) via intraperitoneal injection, perfused transcardially with 0.9% sa-

line (50 ml), followed by 4% paraformaldehyde (50 ml) in phosphate buffer (PB; 0.1M; pH = 7.4). Ages at sacrifice are shown in Table 1. Brains were extracted from the calvaria, postfixed for 24 h in the same fixative, and placed in a 30% sucrose PB solution at 4 °C until sectioning. Each brain was cut in the coronal plane at 40 µm thickness, on a sliding freezing microtome into six adjacent series and stored at 4 °C in a cryoprotectant solution (30 % ethylene glycol, 30 % glycerol, in 0.1 M PB) prior to immunohistochemical staining (Kelley et al., 2014a, 2014b; Velazquez et al., 2013).

Immunohistochemistry

Immunohistochemistry was performed as previously described (Kelley et al., 2014a, 2014b). Tissue was immunostained using the following primary antibodies: a goat polyclonal antibody against choline acetyltransferase ChAT, 1:1000; Millipore, Billerica, MA), a rabbit polyclonal antibody against the pan-neurotrophin receptor $p75^{NTR}$ (1:2000; Millipore), and a rabbit polyclonal antibody against the cognate nerve growth factor (NGF) receptor tyrosine receptor kinase A (TrkA, 1:10,000, gift from Dr. L. Reichardt, UCLA). A 1/6 series of sections was singly immunolabeled for each marker, group sizes are as listed in Table 1. Briefly, sections were washed in 0.1 M phosphate buffer (PB; pH 7.4) to remove cryoprotectant, rinsed in Tris-buffered saline (TBS), and incubated in sodium (meta)periodate (2.139 g per 100 ml TBS) to inhibit endogenous peroxidase activity. To improve primary antibody penetrance throughout the full depth of the tissue, sections was washed in TBS with 0.25 % Triton X-100, followed by rinses in a blocking solution to prevent nonspecific binding consisting of 3 % serum (raised against host organism of secondary antibody: ChAT, horse serum; $p75^{NTR}$ and TrkA, goat serum) in TBS/Triton X-100. Tissue was then incubated overnight at room temperature with the primary

antibody in TBS/Triton X-100 with 1 % serum and then washed in TBS and incubated for 1 h with a biotinylated IgG secondary antibody raised against the host of the primary antibody: ChAT, anti-goat; $p75^{NTR}$, anti-rabbit; TrkA, anti-rabbit (Vector Laboratories, Burlingame, CA). Tissue was washed in TBS and incubated with an avidin-biotin complex (ABC) solution (Elite Kit, Vector Laboratories) for 1 h to amplify the immunochemical reaction. Immunolabeling was visualized using an acetate-imidazole buffer containing 0.05 % 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich, St. Louis, MO) and 0.0015 % freshly prepared H_2O_2. Sections were washed in acetate-imidazole buffer to terminate the immunochemical reaction, mounted onto chrome-alum-subbed slides, air dried for 24 h, dehydrated through a series of graded alcohols (70%, 95%, and 100 %), cleared in xylenes, and cover-slipped with distyrene/dibutylphthalate (plasticizer)/xylene (DPX) mounting medium (Kelley et al., 2014a, 2014b; Velazquez et al., 2013).

BFCN nomenclature

The BFCN subregions examined included the medial septum (MS), vertical limb of the diagonal band (VDB), and nucleus basalis of Meynert/substantia innominata (NBM/SI).

Stereology

ChAT-, $p75^{NTR}$-, and TrkA-positive neuron counts were determined in the MS, VDB, and NBM/SI using the optical fractionator, a stereological model 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., Wil-

liston, VT, USA) coupled to a Nikon Optiphot-2 microscope. 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 x 50 μm counting frame, 151 x 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, 1999). Cell density is presented as cells per 1,000,000 μm^3 (Kelley et al., 2014a). Calculation was performed for each animal, prior to group averages. Photomicrographs were taken on a Nikon Optiphot-2 microscope (Tokyo, Japan) connected to Stereo Investigator software (MicroBrightField, Inc.) Background correction was used at the time of image capture to establish evenness of illumination across the field, and scale bars were added within the Stereo Investigator software. Panels were compiled in PowerPoint (version 14.0.6129.5000, 32-bit, Microsoft, Redmond, WA, USA) and each micrograph was equally corrected for brightness and contrast. No retouching or further manipulations were performed.

Regional area analysis

Regional basal forebrain areas (μm^2) were derived by planimetry. Previous comparison between Cavalieri estimator and planimetry values showed no difference between methods (Kelley et al., 2014a: slope m = 1.001, correlation $r^2 = 0.998$, $r = 0.999$). Values represent summation from tracings outlining each basal forebrain subfield across a 1/6 series: an average of four MS sections, four VDB sections, and seven NBM/SI sections. An area estimate for each basal forebrain subregion was calculated by averaging measures for ChAT- and $p75^{NTR}$-immunolabeled tissue prior to deriving group averages.

Neuron size

BFCN size 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).

Antibody tissue penetration

A major criterion for the use of the optical disector is antibody penetration through the full depth of the stained section. The depth of ChAT, $p75^{NTR}$, and TrkA antibody penetration through a tissue section in the z-axis was determined using the same optical disector system and software used to count labeled neurons in this study (see above). ChAT, $p75^{NTR}$, and TrkA anti-bodies penetrated the full depth of the section allowing for the equal probability of counting all objects, a prerequisite for stereology (Mufson et al., 2000; Kelley et al., 2014a, 2014b; Ve-lazquez et al., 2013).

Statistical Analyses

Statistical and correlation analyses were performed using the Statistical Analysis System (version 9.3; SAS Institute, Cary, NC). The statistical procedure used for each outcome measure is presented below.

HP and VP tasks

For both the HP and VP tasks, the dependent measure for each mouse was the mean number of errors committed per trial, averaged for each block of sessions. For the HP task, the 15 sessions were divided into five 3-day blocks for analysis; for the VP task, the 6 sessions were divided into three 2-day blocks. The data were analyzed using a generalized linear mixed model (PROC GLIMMIX), an established method for conducting repeated measures analyses with various probability distributions including Gaussian (Wolfinger and O' Connell, 1993). Fixed factors for the models included Genotype (Ts65Dn and 2N), Maternal Diet (diet containing normal levels of choline or choline-supplemented), and Cohort (cohorts 1 and 2), as well as appropriate interaction terms. The random factors in the model included Session-Block (defined above) and individual mouse performance. If the interaction between Genotype and Maternal Diet was significant, three post-hoc contrasts of interest were examined: (1) Comparison of unsupplemented Ts65Dn and 2N mice to determine the effect of genotype for mice born to dams maintained on a diet with normal choline levels; (2) Comparison of Ts65Dn mice born to unsupplemented dams versus supplemented dams to determine the effect of MCS for Ts65Dn mice; and (3) Comparison of supplemented 2N mice to unsupplemented 2N mice to determine the effects of MCS for the 2N mice. A Bonferroni correction was applied to these three comparisons, yielding an alpha level of 0.05/3 or 0.0167 as the threshold for significance. If the interaction was not significant, only the main effects of Maternal Diet and/or Genotype are reported.

Morphometric analyses

Analyses of variance (ANOVA) assessed group differences in mean number, density, and size of neurons reactive for ChAT, $p75^{NTR}$, and TrkA within each BFCN region (MS, VDB,

NBM/SI) as described previously (Kelley et al., 2014a). Significant interactions between Maternal Diet and Genotype ($p = 0.05$) were followed up with the three comparisons of interest listed above using a Bonferroni correction.

Group differences in regional area of the various BFCN regions were assessed using the non-parametric Wilcoxon rank sum because normality assumptions of ANOVA were not met.

Correlations between behavioral and morphometric data

A non-parametric Spearman rank correlation was used to assess the relationship between each of the morphometric indices for the MS [count, density, and neuron size for each marker (ChAT, $p75^{NTR}$, TrkA)], and the mean number of errors committed in block 3 of the HP task, the block of testing sessions in which the largest group differences were observed. The use of a non-parametric test mitigated the influence of a few outliers.

RESULTS

Sample size

The final sample size for all behavioral and morphometric measures is presented in Table 1. The four treatment groups were: 1) disomic mice born to dams maintained on a choline normal diet (2N Ch); (2) Ts65Dn mice born to dams maintained on a choline normal diet (Ts Ch); (3) disomic mice born to dams maintained on a choline supplemented diet (2N Ch+); and (4) Ts65Dn mice born to dams maintained on a choline supplemented diet (Ts Ch+). Note that the final sample size for the TrkA endpoints was smaller than for the other analyses due to tissue labeling variability.

Body Weight

Analysis of body weight at the start of testing revealed a main effect of Genotype ($F_{(1,69)} = 16.61$, $p < 0.0001$) but no effect of Maternal Diet ($F_{(1,69)} = 0.35$, $p = 0.556$), and no interaction of Genotype and Maternal Diet ($F_{(1,69)} = 0.09$, $p = 0.769$). The average body weight of trisomic mice was 9% lower than the 2N mice (means ± S.E.M: 2N mice: 35.2 ± 0.50; Ts65Dn mice: 32.18 ± 0.55), consistent with prior reports (Bianchi et al., 2010; Fuchs et al., 2012; Roper et al., 2006; Velazquez et al., 2013).

BFCN Subregions

The nomenclature and topography of the mouse BFCN subfields are in accordance with earlier studies by our collaborative group (Kelley et al., 2014a; 2014b). BFCNs of the MS, VDB and NBM/SI appear as small, spherical and ovoid ChAT-immunoreactive neurons concentrated along the midline with extensions ventrolaterally between the subcallosal region, rostrally, and hemispheric crossing of the anterior commissure, caudally. More caudally, a diffuse group of large multipolar cholinergic neurons are located along the ventromedial aspect of the globus pallidus, which correspond to the NBM/SI (see Supplementary Fig. A.1 – A.3).

Table 1: Age and treatment groups

Group[a]	Age range & mean age at sacrifice	Final sample size (Mean age in months)			
		RAWM	ChAT [b]	p75^{NTR} [b]	TrkA [b]
2N					
Ch	13.7–17.8 (15.6)	19 (15.6)	18 (15.6)	15-20 (15.6)	7-12 (15.5)
Ch+	13.7–17.6 (15.5)	20 (15.5)	19 (15.4)	15-18 (15.5)	9-13 (15.0)
Ts65Dn					
Ch	13.7–17.4 (15.3)	13 (15.3)	15 (15.4)	14-16 (15.3)	4-8 (15.3)
Ch+	13.8–17.6 (15.7)	21 (15.7)	21-22 (15.8)	20-23 (15.7)	9-14 (15.7)

[a] 2N: disomic wildtype mice whose dams were provided either a choline normal (Ch) or choline supplemented (Ch+) diet; Ts65Dn mice whose dams were provided either a choline normal (Ch) or choline supplemented (Ch+) diet. Tissue immunolabeled for antibodies: ChAT: choline acetyltransferase, p75^{NTR}: pan-neurotrophin receptor protein 75 KDa, TrkA: tyrosine kinase receptor A.

Effects of genotype and MCS on HP and VP task performance

Analysis of mean errors in the HP task revealed a main effect of Genotype ($F_{(1, 101.5)}$ = 20.77, $p < 0.0001$). Although the main effect of Maternal Diet was not significant ($F_{(1, 101.5)}$ = 2.43, $p = 0.122$), there was a significant interaction of Genotype and Maternal Diet ($F_{(1, 101.5)}$ = 5.11, $p = 0.026$). Specifically, Ts65Dn mice born to dams on the unsupplemented diet committed a significantly higher number of errors than their 2N counterparts ($p < 0.0001$, Fig. 1A). Supplementing the maternal diet with additional choline significantly improved performance of the Ts65Dn offspring relative to Ts65Dn offspring born to dams on the unsupplemented diet (p = 0.012, Fig. 1A). The relationship between the groups did not vary by session-block (Fig. 1B). MCS did not significantly affect performance of the 2N mice on this task.

In the VP task, neither the main effect of Genotype ($p > 0.20$), the main effect of Maternal Diet ($p > 0.20$) nor the interaction of Genotype and Maternal Diet ($p > 0.20$) was significant. Although a significant interaction was detected between Genotype and Session-block ($F_{(2, 68.72)}$ = 4.61; $p = 0.013$), the two genotypes did not differ significantly in any session-block (Fig 1C).

Effects of genotype and MCS on BFCN density

In the MS, there was a significant interaction between Genotype and Maternal Diet for ChAT-positive neuron density ($F_{(1,70)}$ = 2.70, $p = 0.017$). MS ChAT-positive neuron density was significantly reduced by 18% for unsupplemented Ts65Dn offspring relative to their 2N counterparts ($p = 0.008$, Fig. 2A). MCS significantly increased the density of MS ChAT-positive neurons by 17% for Ts65Dn mice ($p = 0.036$, Fig. 2A), achieving a level comparable to unsupplemented 2N mice. Maternal diet did not affect this measure for the 2N mice.

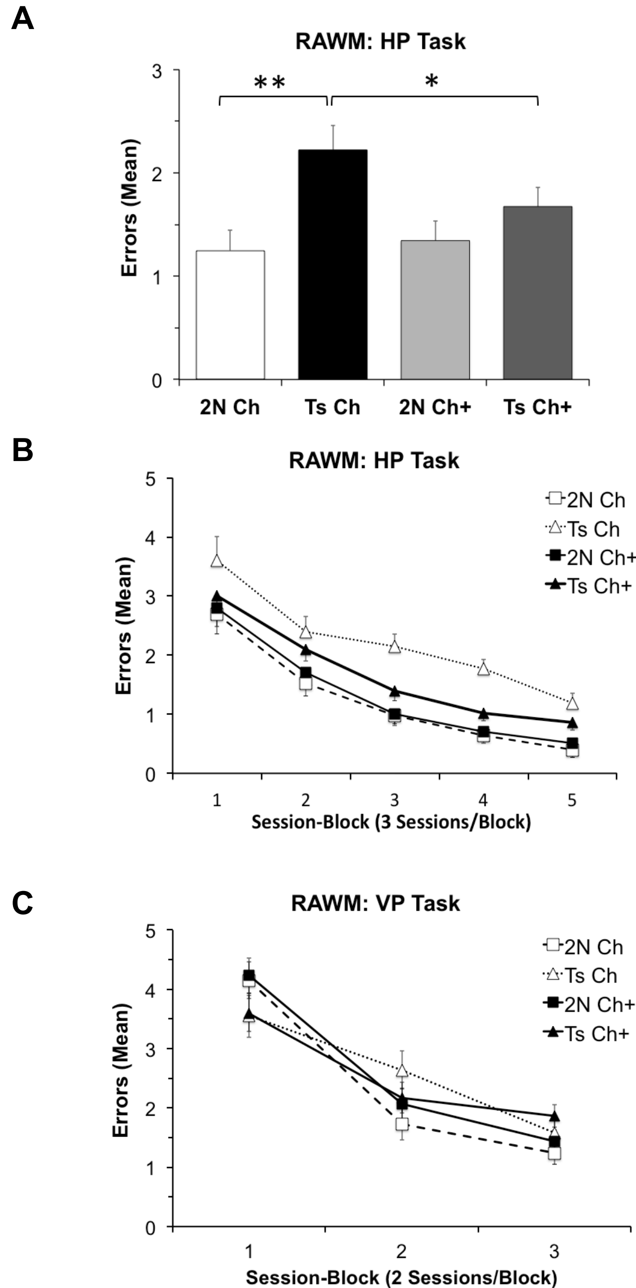

Figure 1. RAWM performance: **(A)** Average errors per trial (collapsed across sessions) in the HP Task was significantly higher for the unsupplemented Ts65Dn mice than their 2N counterparts. MCS significantly improved performance for Ts65Dn (p = 0.011) but not 2N mice; (**B)** Mean errors per trial across the 15 sessions of the HP task; (**C)** Mean errors in the VP task across the 6 sessions on this task; no significant groups differences were seen. **Abbreviations**: 2N Ch: disomic mice born to dams maintained on a choline normal diet (n = 19); Ts Ch: Ts65Dn mice born to dams maintained on a choline normal diet (n = 13); 2N Ch+: disomic mice born to dams maintained on a choline supplemented diet (n = 20); Ts Ch+: Ts65Dn mice born to dams maintained on a choline supplemented diet (n = 21); HP: Hidden platform task of the radial arm watermaze; VP: Visible platform task of the radial arm watermaze; MCS: maternal choline supplementation; * $p \leq 0.05$, ** $p \leq 0.001$.

The density of MS $p75^{NTR}$- positive neurons was also significantly lower (13%) for Ts65Dn mice relative 2N counterparts (Main effect of Genotype: $F_{(1,73)} = 16.16$, $p = 0.0001$, Fig. 2A). This same genotype effect was found for the density of MS TrkA-positive neurons (Main effect of Genotype: $F_{(1,43)} = 10.97$, $p = 0.002$, Fig. 2A). MCS significantly increased the density of $p75^{NTR}$-positive MS neurons by 7% for both genotypes (Main effect of Maternal Diet: $F_{(1,73)} = 4.31$, $p = 0.041$, Fig. 2A), but did not affect TrkA-positive neuron density in this region, indicating a dissociation between NGF receptor phenotypes.

The NBM/SI of Ts65Dn mice showed a significant 22% reduction in the density of $p75^{NTR}$-positive neurons relative to 2N mice (Main effect of Genotype: $F_{(1,64)} = 44.78$, $p < 0.0001$, Fig. 2C) similar to that seen in the MS. MCS had no effect on this measure in the NBM/SI. In contrast to observations within the MS, neither ChAT- nor TrkA-positive neurons were significantly altered by Genotype, Maternal Diet or their interaction, in the NBM/SI.

For the VDB, there was no significant effect of Genotype, Maternal Diet or their interaction for BFCN density, regardless of the markers examined (Fig. 2B).

Effects of genotype and MCS on BFCN cell counts

Genotype did not significantly alter ChAT-positive neuron count in any of the three regions examined. MCS did alter the number of ChAT-positive BFCN neurons, but the effect varied by subregion and genotype. In the MS, an interaction between Genotype and Maternal Diet was seen ($F_{(1, 70)} = 4.63$, $p = 0.035$). MCS produced a significant 30% increase in the number of MS ChAT-positive neurons in Ts65Dn mice (Ts65Dn Ch vs. Ts65Dn Ch+; $p = 0.009$, Fig. 3A), but not 2N mice ($p > 0.20$). In the VDB, MCS produced a significant 20% increase in the num-

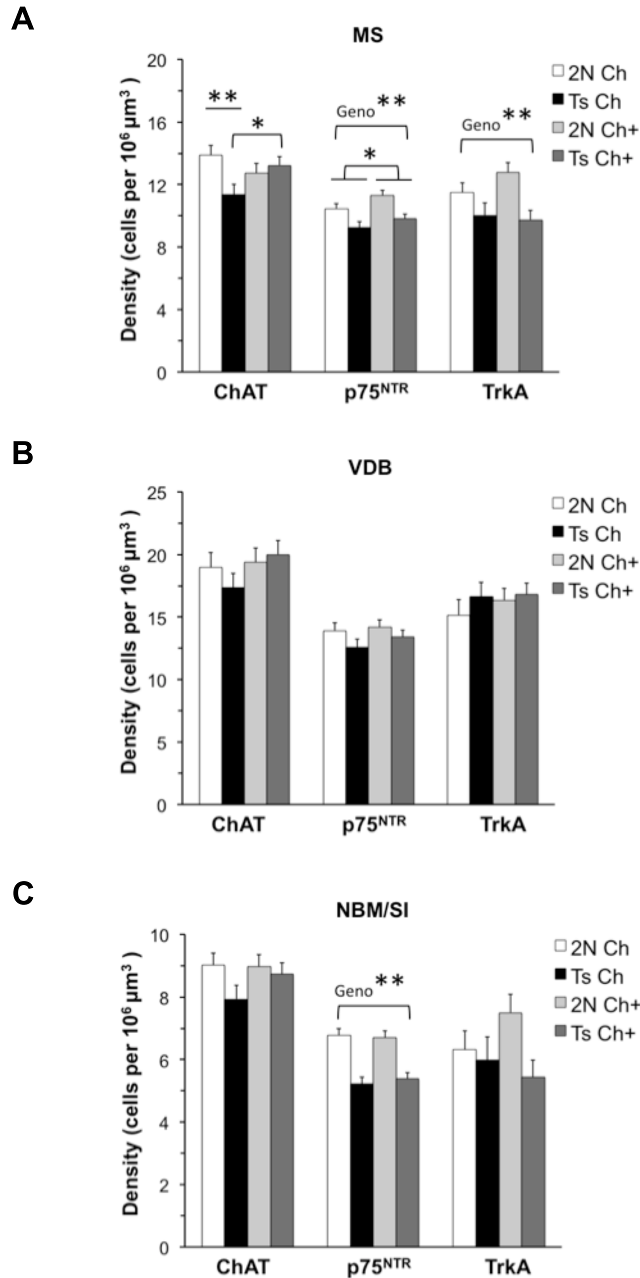

Figure 2. Density of ChAT-, p75^{NTR}-, and TrkA-positive cells in the basal forebrain: (A) In the MS, Ts Ch mice showed a significantly lower ChAT-positive density relative to 2N Ch mice (p = 0.008), whereas both groups of Ts65Dn mice showed reduced density of p75^{NTR}-positive and TrkA-positive BFCNs relative to 2N mice (p75^{NTR}: p = 0.0001; TrkA: p = 0.002). MCS significantly increased the density of ChAT-positive BFCNs in Ts65Dn mice (p = 0.036); **(B)** No significant differences were seen in the VDB; **(C)** In the NBM/SI, both groups of Ts65Dn mice showed reduced p75^{NTR}-positive BFCN density relative to 2N mice (p < 0.0001). **Abbreviations**: 2N Ch: disomic mice born to dams maintained on a choline normal diet; Ts Ch: Ts65Dn mice born to dams maintained on a choline normal diet; 2N Ch+: disomic mice born to dams maintained on a choline supplemented diet; Ts Ch+: Ts65Dn mice born to dams maintained on a choline supplemented diet; Geno: Main effect of Genotype; MS: Medial Septum; VDB: Ventral Diagonal Band; NBM/SI: Nucleus Basalis of Meynert/Substantia Innominata; BFCNs: basal forebrain cholinergic neurons; ChAT: Choline Acetyltransferase; p75^{NTR}: pan neurotrophin receptor; TrkA: tyrosine kinase A receptor; MCS: maternal choline supplementation;*p ≤ 0.05, **p ≤ 0.001.

-ber of ChAT-positive neurons, with a comparable effect seen for the two genotypes (Main effect of Maternal Diet: $F_{(1, 70)} = 5.58$, $p = 0.021$, Fig. 3B). A similar but non-significant 9% increase in ChAT-positive neurons was seen in the NBM/SI for both genotypes (Main effect of Maternal Diet: $F_{(1, 68)} = 2.30$, $p = 0.134$, Fig. 3C).

The number of $p75^{NTR}$ immunoreactive neurons was significantly reduced (23%) in trisomic mice relative to their 2N counterparts in the NBM/SI (Main effect of Genotype: $F_{(1,64)} = 17.61$, $p < 0.0001$; Fig. 3C), with no Genotype effect for the MS (Fig. 3A) or VDB (Fig. 3B). MCS did not significantly affect the number of $p75^{NTR-}$ labeled neurons for either genotype in any BFCN subregion examined (Fig. 3A-C).

The number of TrkA-positive neurons was significantly higher in Ts65Dn mice than their 2N counterparts in the VDB (Main effect of Genotype: $F_{(1, 37)} = 4.72$, $p = 0.036$; Fig 3B). In contrast, the genotypes did not differ significantly for TrkA-positive neuron count in the MS ($p = 0.103$, Fig. 3A) or NBM/SI ($p = 0.150$, Fig. 3C). MCS did not significantly alter TrkA-positive neuron counts for either genotype in any region examined.

Effects of genotype and MCS on BFCN size

ChAT-, $p75^{NTR}$-, or TrkA-positive neuron size did not differ between the two genotypes in any region examined. MCS altered BFCN size only in the MS, producing a significant 15% increase in TrkA-positive neuron size in this region, with a comparable effect seen in both genotypes (Main effect of Maternal Diet: $F_{(1, 42)} = 3.89$, $p = 0.050$, Fig. 4A). MCS did not alter the size of TrkA- positive neurons in the VDB (Fig. 4B) or NBM/SI (Fig. 4C), nor ChAT- or $p75^{NTR}$-positive neurons in any of the three subregions examined (Fig. 4A-C).

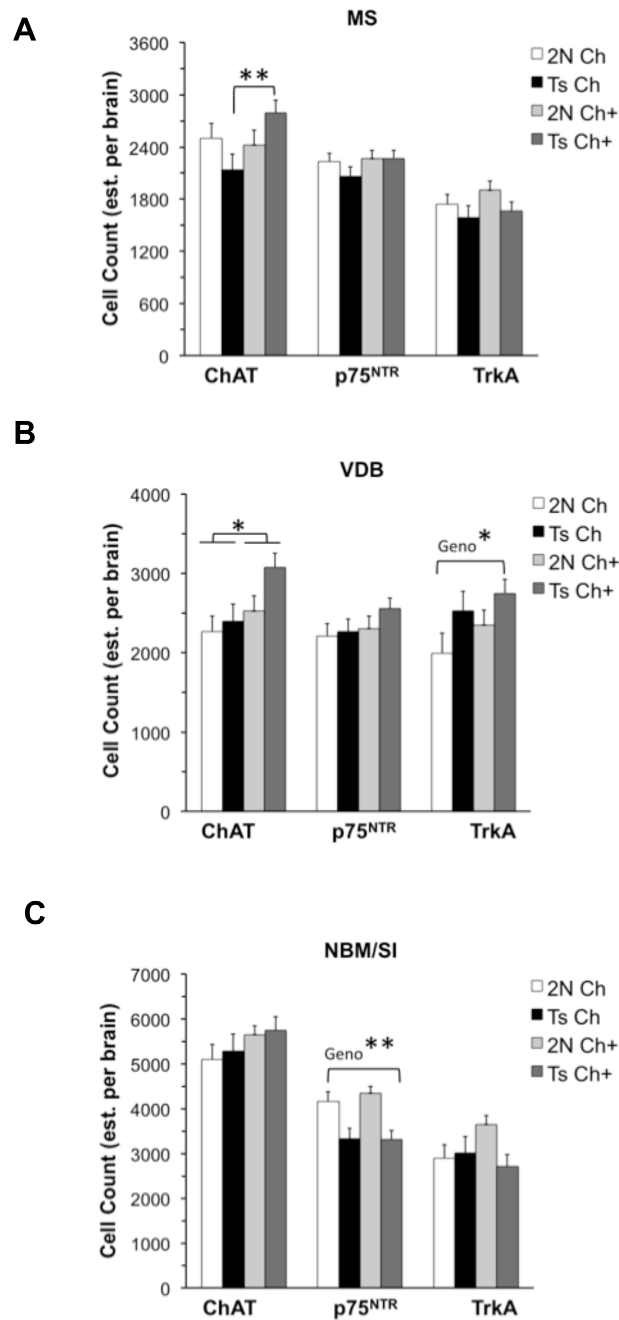

Figure 3. Number of ChAT-, p75^{NTR}-, and TrkA-positive BFCNs in the basal forebrain: (A) In the MS, MCS significantly increased the number of ChAT-positive BFCNs in Ts65Dn mice (*p* = 0.009); **(B)** In the VDB, both groups of Ts65Dn mice showed a significantly higher number of TrkA-positive BFCNs (*p* = 0.036). MCS increased the number of ChAT-positive BFCNs for both genotypes (*p* = 0.021); **(C)** In the NBM/SI, Ts65Dn mice exhibited a significantly higher number of p75^{NTR}-positive BFCNs than the 2N mice ($p < 0.0001$). **Abbreviations**: 2N Ch: disomic mice born to dams maintained on a choline normal diet; Ts Ch: Ts65Dn mice born to dams maintained on a choline normal diet; 2N Ch+: disomic mice born to dams maintained on a choline supplemented diet; Ts Ch+: Ts65Dn mice born to dams maintained on a choline supplemented diet; Geno: Main effect of Genotype; MS: Medial Septum; VDB: Ventral Diagonal Band; NBM/SI: Nucleus Basalis of Meynert/Substantia Innominata; BFCNs: basal forebrain cholinergic neurons; ChAT: Choline Acetyltransferase; p75^{NTR}: pan neurotrophin receptor; TrkA: tyrosine kinase A receptor; MCS: maternal choline supplementation; * $p \leq 0.05$, ** $p \leq 0.001$.

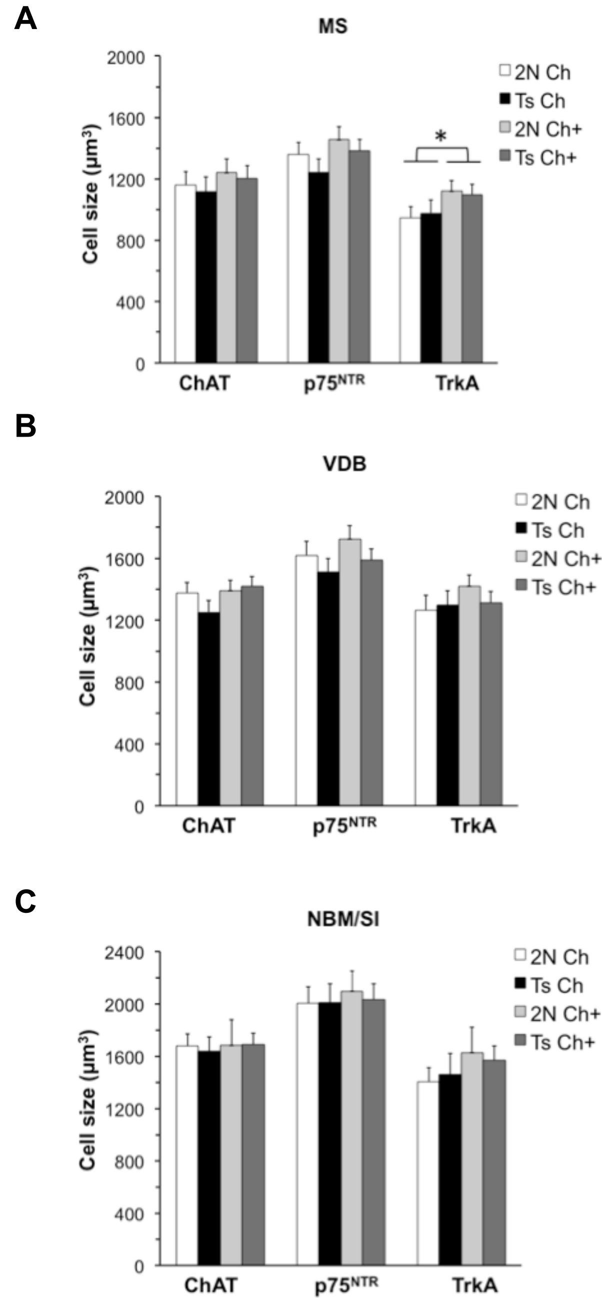

Figure 4. Size of ChAT-, $p75^{NTR}$-, and TrkA-positive cells in the basal forebrain: (A) In the MS, MCS increased TrkA-positive BFCNs for both genotypes ($p = 0.050$); There was no effect of genotype, maternal diet, nor their interaction in **(B)** the VDB, or **(C)** the NBM/SI. **Abbreviations:** 2N Ch: disomic mice born to dams maintained on a choline normal diet; Ts Ch: Ts65Dn mice born to dams maintained on a choline normal diet; 2N Ch+: disomic mice born to dams maintained on a choline supplemented diet; Ts Ch+: Ts65Dn mice born to dams maintained on a choline supplemented diet; MS: Medial Septum; VDB: Ventral Diagonal Band; NBM/SI: Nucleus Basalis of Meynert/Substantia Innominata; BFCNs: basal forebrain cholinergic neurons; ChAT: Choline Acetyltransferase; $p75^{NTR}$: pan neurotrophin receptor; TrkA: tyrosine kinase A receptor; MCS: maternal choline supplementation; * $p \leq 0.05$, ** $p \leq 0.001$.

Effects of genotype and MCS on region area

Region area of the VDB and NBM/SI was significantly larger in the unsupplemented Ts65Dn mice relative to their 2N counterparts (Wilcoxon Rank Sum Test, VDB: $p = 0.016$, Fig. 5B; NBM/SI: $p = 0.033$; Fig. 5C). In contrast, MS region area did not vary by genotype (Fig. 5A). The effect of MCS on region area varied by genotype and the specific subregion evaluated. MCS led to a significant 8% average decrease in MS area for 2N mice (Wilcoxon Rank Test, $p = 0.046$, Fig. 5A), with no effect for the VDB or NBM/SI. There was no effect of MCS on region area for Ts65Dn mice for any subfield examined (Fig. 5A-C).

Correlations between maze performance and BFCN indices

Correlational analyses revealed a significant negative association between the mean number of errors in block 3 of the HP task and density of ChAT-positive MS neurons ($r_s = -0.31$, $p = 0.014$; Fig. 6A). A similar trend was seen for the density of TrkA-positive MS neurons ($r_s = -0.27$, $p = 0.095$; Fig. 6B). There were no significant relationships between maze performance and size of ChAT- or TrkA-positive neurons.

A significant negative correlation was also observed between the mean number of block 3 errors and size of $p75^{NTR}$-positive neurons in the MS ($r_s = -0.26$; $p = 0.036$; Fig. 6C), with a similar trend seen for MS $p75^{NTR}$-positive neuron numbers ($r_s = -0.23$, $p = 0.062$; Fig. 6D).

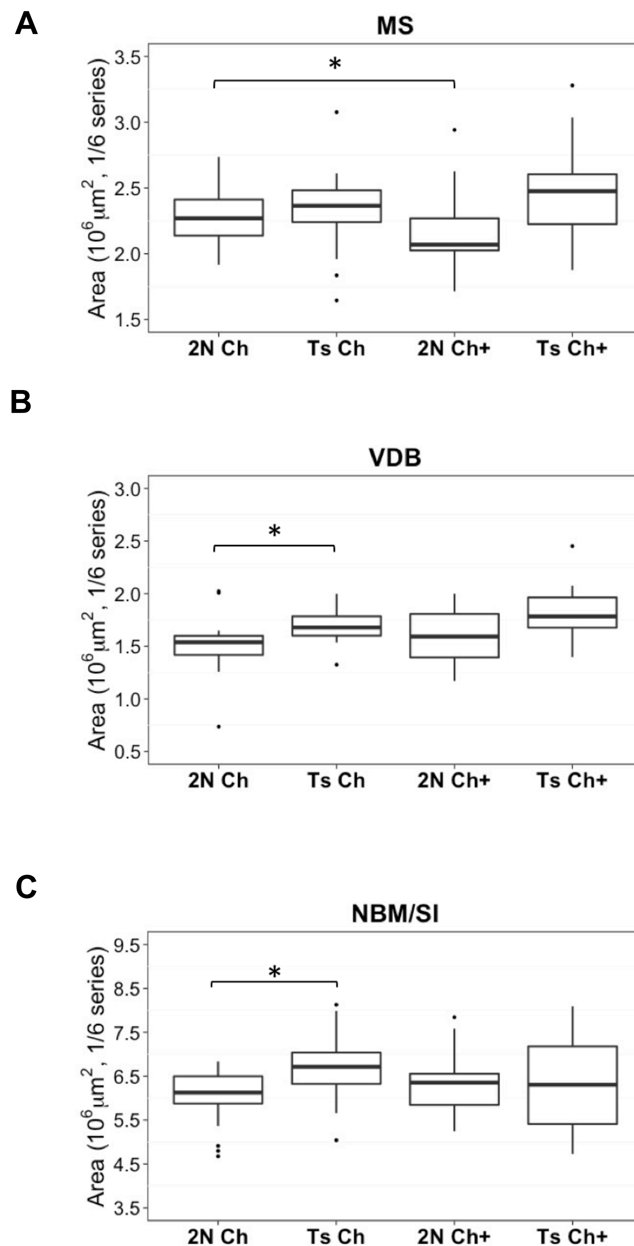

Figure 5. Regional basal forebrain areas: (A) MCS decreased MS area for 2N mice (p = 0.046), but had no effect for Ts65Dn mice; **(B)** Unsupplemented Ts65Dn mice showed significantly larger VDB area than unsupplemented 2N mice (p = 0.016), a pattern also seen for the **(C)** NBM/SI (p = 0.033). MCS had no effect on VDB or NBM/SI for either genotype. **Abbreviations:** 2N Ch: disomic mice born to dams maintained on a choline normal diet; Ts Ch: Ts65Dn mice born to dams maintained on a choline normal diet; 2N Ch+: disomic mice born to dams maintained on a choline supplemented diet; Ts Ch+: Ts65Dn mice born to dams maintained on a choline supplemented diet; MS: Medial Septum; VDB: Ventral Diagonal Band; NBM/SI: Nucleus Basalis of Meynert/Substantia Innominata; BFCNs: basal forebrain cholinergic neurons; ChAT: Choline Acetyltransferase; $p75^{NTR}$: pan neurotrophin receptor; TrkA: tyrosine kinase A receptor; MCS: maternal choline supplementation; * $p \leq 0.05$, ** $p \leq 0.001$.

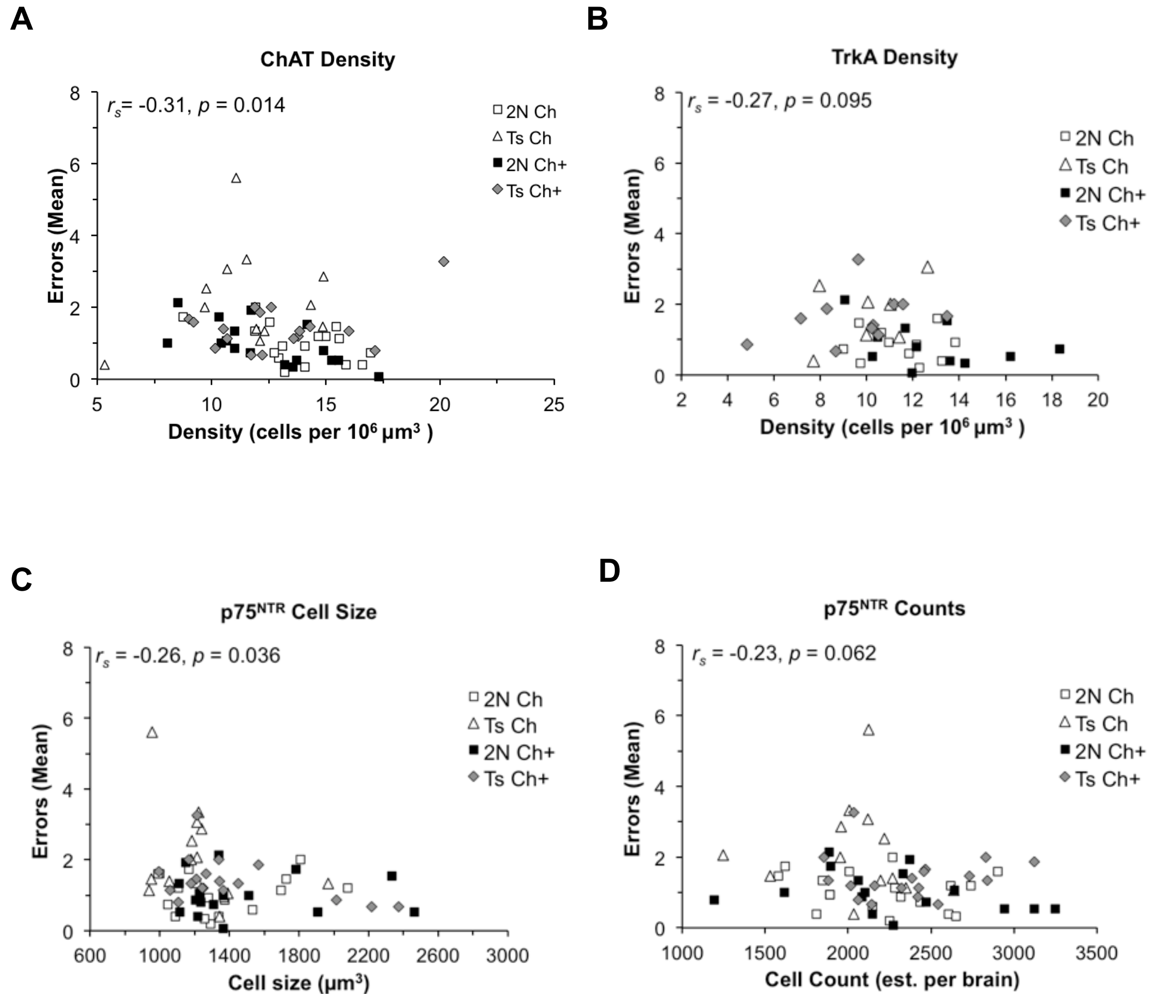

Figure 6. Correlations between HP performance and BFCN indices in the MS: Mean errors in block 3 of the HP task was negatively correlated with **(A)** Density of ChAT-positive BFCNs (p = 0.014), **(B)** Density of TrkA-positive BFCNs (p = 0.095); **(C)** Cell size of $p75^{NTR}$-positive BFCNs (p = 0.036), and **(D)** Number of $p75^{NTR}$- positive BFCNs (p = 0.062). **Abbreviations**: 2N Ch: disomic mice born to dams maintained on a choline normal diet; Ts Ch: Ts65Dn mice born to dams maintained on a choline normal diet; 2N Ch+: disomic mice born to dams maintained on a choline supplemented diet; Ts Ch+: Ts65Dn mice born to dams maintained on a choline supplemented diet; HP: Hidden platform task of the radial arm watermaze; BFCNs: basal forebrain cholinergic neurons; C hAT: Choline Acetyltransferase; $p75^{NTR}$: pan neurotrophin receptor; TrkA: tyrosine kinase A receptor.

DISCUSSION

The present study demonstrated that supplementing the maternal diet with additional choline during pregnancy and lactation significantly improved spatial cognition of Ts656Dn offspring and provided BFCN neuroprotection in a phenotypic- and subregion-specific manner. Moreover, several of these BFCN indices, notably those in the MS, correlated significantly with maze performance, providing powerful support for a functional relationship between these behavioral and morphometric effects of MCS. These data have significant translational potential for minimizing BFCN dysfunction in DS, and add to the growing evidence that maternal choline intake should be increased during pregnancy.

MCS improves spatial cognition of Ts65Dn offspring

Unsupplemented Ts65Dn mice performed significantly less well in the HP task than their 2N littermates. In contrast, these groups did not differ in the VP task, indicating that the impaired performance of the unsupplemented trisomic mice in the HP task cannot be attributed to impairments in visuomotor skills, swimming ability, or motivation to escape from the water. Rather, these data collectively indicate that the poor performance of Ts65Dn mice in the HP task is due to impaired spatial mapping, subserved by the hippocampus, consistent with prior findings with spatial mazes (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, Velazquez et al., 2013), as well as other hippocampal-dependent tasks (Bianchi et al., 2010; Hyde and Crnic, 2001; Hyde et al., 2001; Lockrow et al., 2011).

Importantly, supplementing the maternal diet with additional choline significantly improved performance of the trisomic offspring in the HP task. These results extend our prior find-

ings that MCS improves attention and emotion regulation in Ts65Dn offspring (Moon et al., 2010), functions subserved by BFCN projections to the cortex. A prior study from our lab also demonstrated that MCS normalized hippocampal neurogenesis in Ts65Dn offspring, which correlated with their improved spatial mapping ability (Velazquez et al., 2013). Together, these findings suggest that increased maternal choline intake during pregnancy and lactation results in lasting improvement in cognitive and affective functions in Ts65Dn offspring, perhaps due to the normalization of BFCN projection systems.

MCS did not significantly affect performance of the 2N mice on the spatial learning task. This lack of effect in the 2N mice is likely due to the task not being sufficiently demanding. This inference is based on prior water maze studies, which have shown benefits of MCS for normal rats when the location of the hidden escape platform was changed daily, but not when it remained in the same location across sessions (Tees, 1999; Tees and Mohammadi, 1999), as was the case in the version of the HP task used in the present study. The less demanding reference memory version of the HP task was selected for the present study only after extensive pilot testing demonstrated that the Ts65Dn mice could not solve the more demanding explicit memory version of this task.

MCS protects MS BFCNs in Ts65Dn offspring

We observed differences in BFCN number and density between unsupplemented trisomic and unsupplemented 2N mice, which varied by BFCN subregion and phenotypic marker. In the MS, the density of ChAT- and $p75^{NTR}$-immunoreactive neurons was significantly reduced in the unsupplemented trisomic mice relative to their 2N counterparts. A similar, albeit non-significant, pattern was seen for neuron number in this region consistent with previous reports using Ts65Dn

mice in the age-range examined here (Contestible et al., 2006; Cooper et al., 2001; Hunter et al., 2001; Salehi et al., 2006; Seo and Isacson, 2005). The Ts65Dn mice also show a significant reduction in TrkA-positive BFCNs in the MS relative to their 2N littermates; no prior studies have reported on this measure in mid-life.

In the NBM/SI, the trisomic mice exhibited significantly reduced number and density of $p75^{NTR}$-immunoreactive neurons relative to 2N mice, but no differences for ChAT- or TrkA-immunoreactive neurons. In the VDB, there were no genotype differences in the number of ChAT- or $p75^{NTR}$-immunoreactive neurons, commensurate with earlier studies using 18-19 month old mice (Contestible et al., 2006; Hunter et al., 2004). However, Ts65Dn mice exhibited a significantly higher number of VDB TrkA-immunoreactive neurons than 2N mice. These findings are in contrast to reports of reduced numbers of TrkA-positive BFCNs in the MS and VDB of younger (6-10 mos) and older (19-22 mos) Ts65Dn mice relative to 2N mice (Granholm et al., 2000; Lockrow et al., 2011, 2009; Salehi et al., 2006).

MCS normalized select BFCN phenotypes, with the greatest effect on MS neurons. Ts65Dn mice born to supplemented dams had a significantly higher number and density of ChAT-positive neurons within the MS than their unsupplemented counterparts. Moreover, MCS increased the density of $p75^{NTR}$-containing neurons in the MS, and the number of ChAT-positive neurons in the VDB for both genotypes. Although, neither count nor density of these neurons was significantly affected by MCS in the NBM/SI, the patterns across groups were similar. Taken together, these data collectively suggest that MCS has beneficial effects on cholinotrophic BFCN subtypes. The regional variability of MCS effects may be related to differences in BFCN target-derived support related to its survival neurotrophin, NGF, and/or the differences in the specific projection sites of each BFCN subregion. In this regard, the MS and VDB project to the

hippocampus, whereas the NBM/SI projects to the neocortex (Rye et al., 1984, Mesulam et al., 1983).

In contrast to the lower number and density of ChAT- and p75^{NTR}-positive BFCNs for unsupplemented Ts65Dn mice, the size of these neurons did not vary by genotype within any basal forebrain subregion examined. Previous findings indicate atrophy for both ChAT- and p75^{NTR}-immunolabeled BFCNs in the MS and VDB for Ts65Dn mice at younger (6-10 mos) and older (18-22 mos) ages (Lockrow et al., 2011; Salehi et al., 2006; Cooper et al., 2001; Granholm et al., 2000; Holtzman et al., 1996). However, similar to the current findings, no differences between genotypes were reported for BFCN cell size in mice 11 to 14 months of age (Granholm et al., 2002; Seo and Isacson, 2005). These findings suggest an overall age-dependent atrophy of BFCNs in unsupplemented trisomic mice during early development and advanced age, but normal cell size during mid-life. These results imply either an increase in cell-size for trisomic mice from development to mid-adult life, possibly as a neuroplastic compensation for a frank loss in number and density of cholinergic neurons, or alternatively degenerative, neuronal atrophy in 2N mice from development to mid-life.

Supplementing the maternal diet with additional choline significantly increased the size of TrkA-positive MS neurons in both Ts65Dn and 2N offspring. Similar patterns, albeit non-significant, were seen for the size of ChAT- and p75^{NTR}-positive neurons in this region. A prior study reported that MCS significantly increased p75^{NTR}-positive cell size in the MS/VDB at 6 to 7 months of age in normal rats, relative to rats born to dams on a standard choline diet (Williams et al., 1998), but no studies to date have examined the effects of this maternal choline dietary intervention on the size of TrkA-positive neurons. The increase in TrkA-immunoreactive cell size in the MS suggests upregulation of the NGF cognate cell survival receptor (Bothwell,

1995; Hempstead, 2006). These findings lend further support to the concept that MCS has a complex, heterogeneous effect on intracellular mechanisms and signaling pathways associated with cholinotrophic neuronal activity, which our morphometric data suggests may be region- and age-dependent.

Possible Mechanisms

The current investigation demonstrated that supplementing the maternal diet with additional choline improved spatial cognition in the Ts65Dn offspring and offered some protection to BFCNs, with the greatest effect on those within the MS, which provide the primary cholinergic innervation to the hippocampus (Mesulam et al., 1983). Importantly, errors in the maze task were significantly (inversely) correlated with the density of ChAT- and TrkA-positive neurons in the MS as well as the number and size of $p75^{NTR}$- positive neurons in the MS. The data reported herein suggest that the normalization of MS cholinergic hippocampal projection neurons in Ts65Dn mice due to MCS contributes to their improved spatial cognition. Our group has also shown that MCS partially normalized adult hippocampal neurogenesis in trisomic offspring relative to their unsupplemented counterparts, and that the degree of neurogenesis was a significant predictor of water maze performance (Velazquez et al., 2013). These findings suggest that both of these effects of MCS (increased hippocampal neurogenesis and BFCN neuroprotection) contribute to improved spatial cognition in supplemented Ts65Dn mice.

The protection of BFCNs in Ts65Dn mice may reflect the effects of increased maternal choline intake on neurotrophic factors. For example, normal adult rat offspring of choline-supplemented dams exhibit increased brain levels of NGF and brain-derived neurotrophic factor (BDNF) relative to those born to unsupplemented dams (Glenn et al., 2007; Sandstrom et

al., 2002). Based on these data, it is reasonable to posit that MCS enhances target-derived neuroprotection of Ts65Dn BFCNs, which typically begin to atrophy at six months of age due to impaired retrograde transport of NGF (Cooper et al., 2001; Granholm et al., 2000; Holtzman et al., 1996, 1992; Salehi et al., 2006). The resulting increased functionality of these neurons would then contribute to improvement in various cognitive tasks, especially related to BFCN projection systems, notably the hippocampus and frontal cortex.

Although much remains to be learned regarding the specific mechanism(s) by which supplementing the maternal diet with additional choline exerts life-long effects on offspring cognitive functioning, BFCN projection neurons, hippocampal neurogenesis and neurotrophins, all of these effects likely reflect one or both of two broad categories of effects: First, these effects may be due to organizational brain changes, secondary to choline's role as the precursor to phosphatidyl choline, a major constituent of cellular membranes, and its role as the precursor of acetylcholine, an important ontogenetic signal (Cermak et al., 1999; Meck et al., 1989; Zeisel and Niculescu, 2006). Second, these effects may be related to 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, 2009). Choline has a primary role as a methyl donor, through the betaine-methionine pathway, and alterations in dietary levels of choline during early development can produce life-long effects on gene expression through DNA methylation and histone modifications (Blusztajn and Mellot, 2012; Davison et al., 2009).

Increased need for choline during early development

The lifelong beneficial effects of MCS seen in the Ts65Dn offspring in the current study should be interpreted within the context of current choline intake recommendations. Dietary

recommendations for choline, first established in 1998 by the Institute of Medicine, were based on the level of choline needed to prevent liver dysfunction. However, several converging lines of evidence suggest that these recommendations are insufficient during pregnancy to optimize brain development, cognitive functioning, and lifelong health of the offspring. First, data from both animal models and humans indicate that pregnancy causes a pronounced depletion of maternal choline pools indicating that choline requirements during pregnancy are increased and that the need for this nutrient by the fetus may commonly exceed the amount consumed by the mother (Gwee and Sim, 1979; 1978; Yan et al., 2012; Zeisel et al., 1995). Indeed, a doubling of choline intake by pregnant women does not increase the urinary excretion of choline, a water-soluble biomolecule, indicating that the higher intake level does not exceed metabolic requirements (Yan et al., 2012). Second, and perhaps most importantly, numerous rodent studies indicate that supplementing the maternal diet with additional choline produces lifelong beneficial cognitive effects for the offspring and reduces age-related cognitive decline (e.g., McCann et al., 2006; Meck and Williams, 2003; Meck et al., 2008). Finally, consistent with the present findings, MCS not only improves cognitive functioning in normal offspring but also offers protection against a variety of neural insults including those associated with fetal alcohol syndrome (Thomas et al., 2009), Rett syndrome (Nag and Berger-Sweeney, 2007), epilepsy (Holmes et al., 2002; Wong-Goodrich et al., 2008), and schizophrenia (Stevens et al., 2008), providing additional support for increasing maternal choline intake during pregnancy.

Conclusions

The present behavioral and morphometric findings indicate that increasing maternal choline intake normalizes BFCNs in the Ts65Dn mouse model of DS in a complex, phenotype- and

subregion-specific manner. Support for the functional effects of these changes is provided by powerful correlative analyses, which link select BFCN changes to improved performance in a task subserved by the septo-hippocampal system. These data suggest that increasing maternal choline intake during pregnancy may represent a safe and effective therapy for expectant mothers carrying a DS fetus, as well as possibly provide BFCN neuroprotection during aging for the population at large.

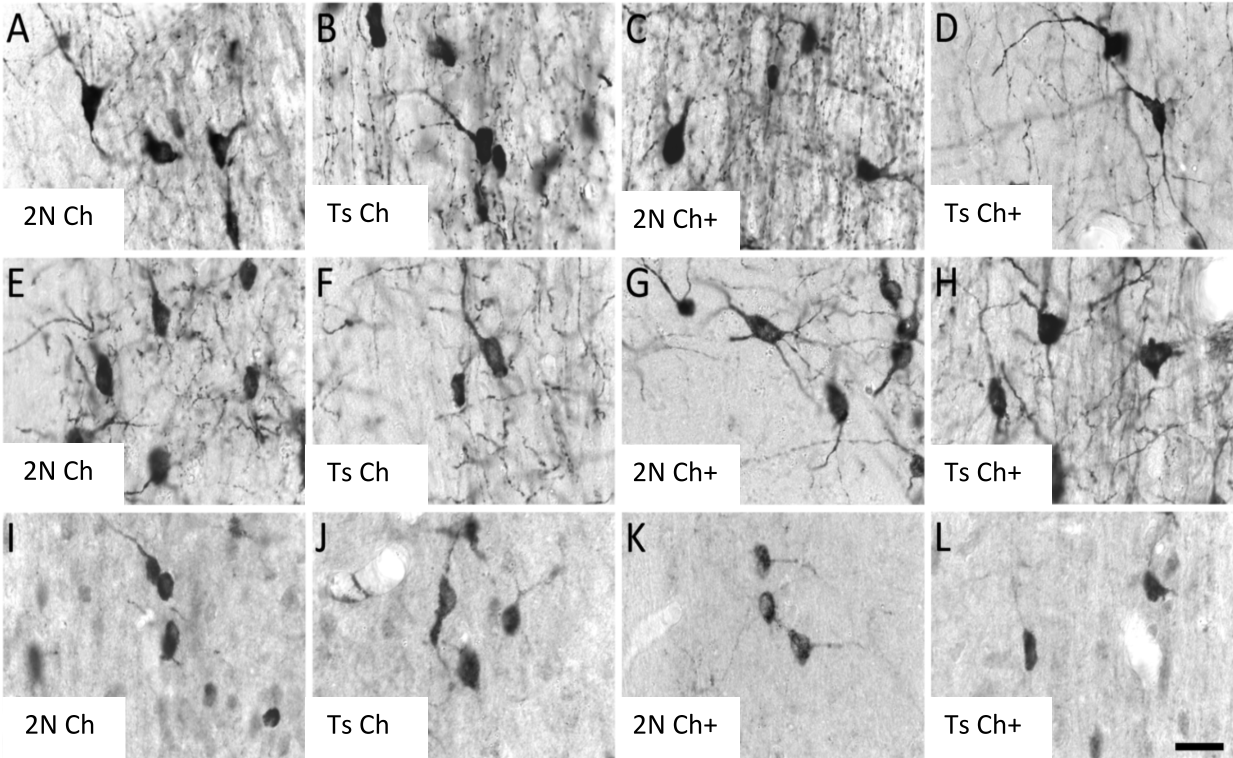

Figure. A.1 Photomicrographs of ChAT-, p75^{NTR}-, and TrkA-positive cells in the MS: Photomicrographs show the distribution of ChAT- **(A – D)**, p75^{NTR}- **(E – H)**, and TrkA- **(I – L)** immunoreactivity within the MS for the four groups of mice. **Abbreviations**: 2N Ch: disomic mice born to dams maintained on a choline normal diet; Ts Ch: Ts65Dn mice born to dams maintained on a choline normal diet; 2N Ch+: disomic mice born to dams maintained on a choline supplemented diet; Ts Ch+: Ts65Dn mice born to dams maintained on a choline supplemented diet; MS: Medial Septum.

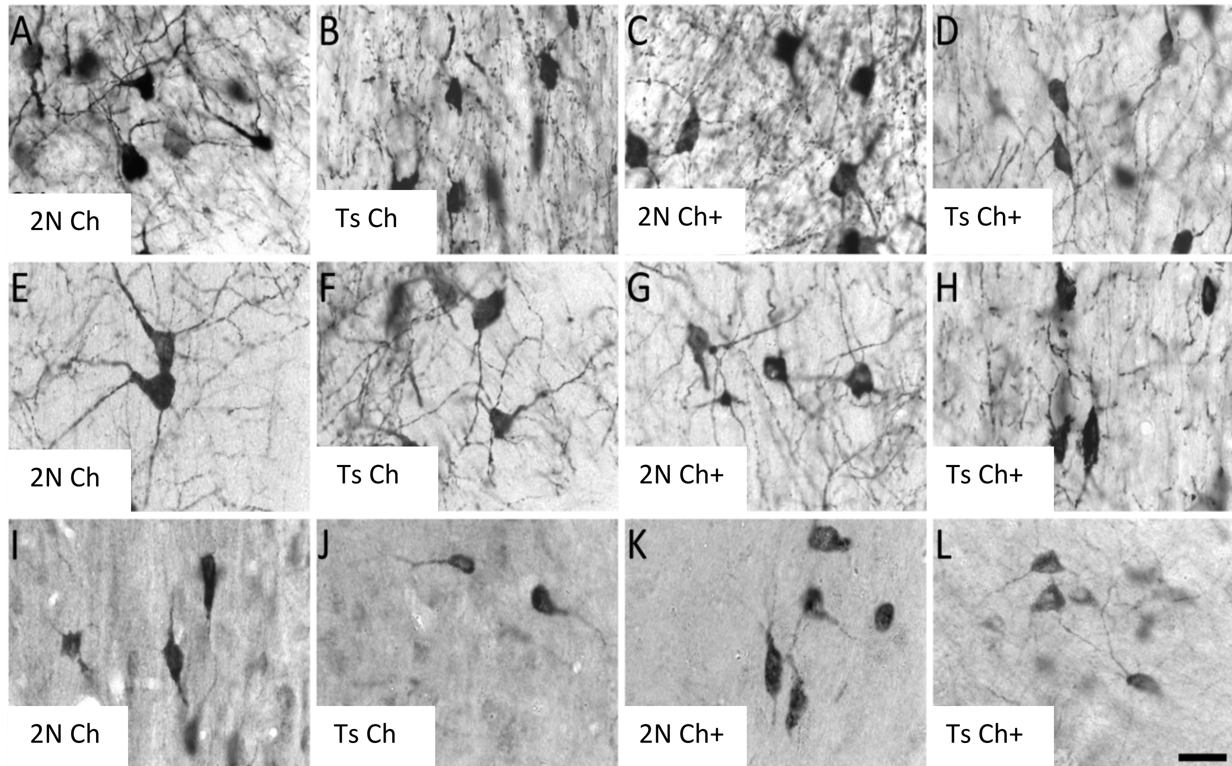

Figure. A.2 Photomicrographs of ChAT-, $p75^{NTR}$-, and TrkA-positive cells in the VDB: Photomicrographs show the distribution of ChAT- **(A – D)**, $p75^{NTR}$- **(E – H)**, and TrkA- **(I – L)** immunoreactivity within the VDB. **Abbreviations**: 2N Ch: disomic mice born to dams maintained on a choline normal diet; Ts Ch: Ts65Dn mice born to dams maintained on a choline normal diet; 2N Ch+: disomic mice born to dams maintained on a choline supplemented diet; Ts Ch+: Ts65Dn mice born to dams maintained on a choline supplemented diet; VDB: Ventral Diagonal Band.

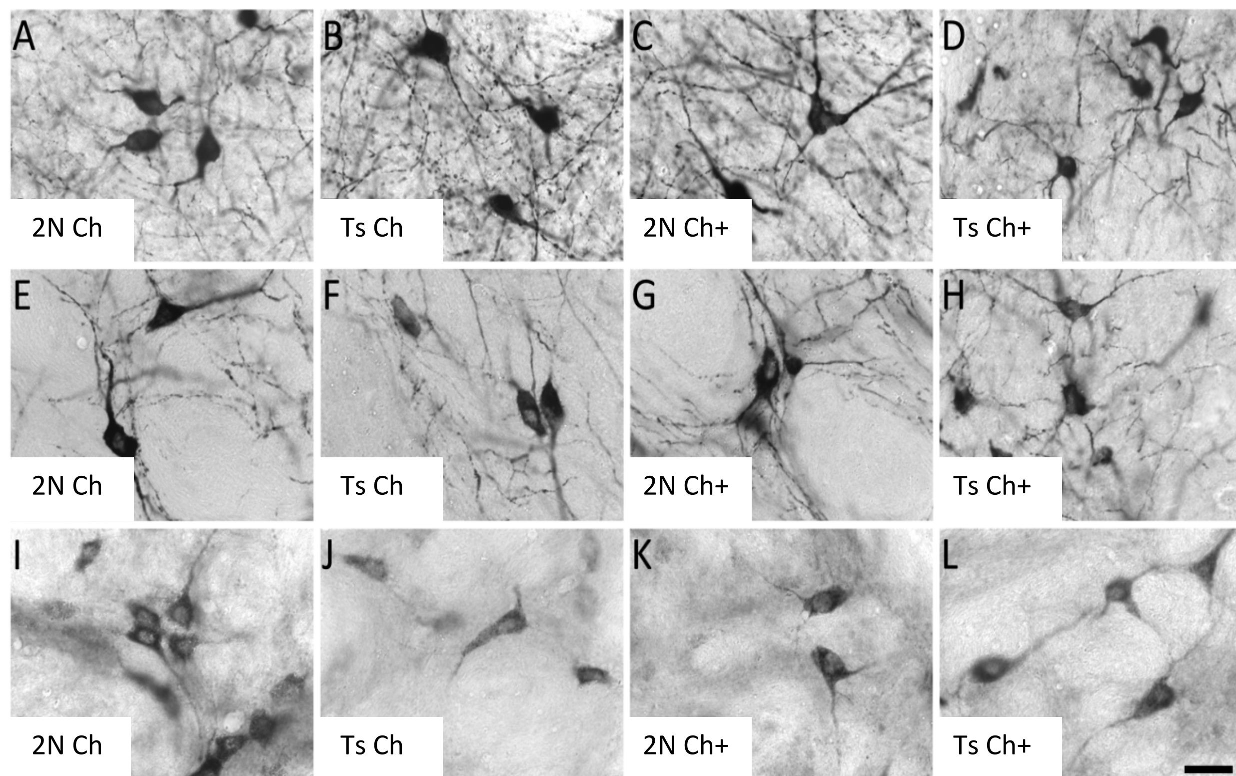

Figure. A.3 Photomicrographs of ChAT-, p75^{NTR}-, and TrkA-positive cells in the NBM/SI: Photomicrographs show the distribution of ChAT- **(A – D)**, p75^{NTR}- **(E – H)**, and TrkA- **(I – L)** immunoreactivity within the VDB. **Abbreviations**: 2N Ch: disomic mice born to dams maintained on a choline normal diet; Ts Ch: Ts65Dn mice born to dams maintained on a choline normal diet; 2N Ch+: disomic mice born to dams maintained on a choline supplemented diet; Ts Ch+: Ts65Dn mice born to dams maintained on a choline supplemented diet; NBM/SI: Nucleus Basalis of Meynert/Substantia Innominata.

CHAPTER 3

Assessment of age-related attention dysfunction in the Ts65Dn mouse model of Down syndrome and Alzheimer's disease

ABSTRACT

In addition to intellectual disability (ID), individuals with Down syndrome (DS) also develop Alzheimer's disease (AD)-like neuropathology during the third to fourth decade of life. The Ts65Dn mouse model of DS exhibits key features of these disorders, notably, impairments in learning, attention, and memory, as well as deficits in development neurogenesis and loss of basal forebrain cholinergic neurons (BFCNs) in the medial septum (MS). The present study was designed to characterize cholinergic neurons in the nucleus basalis of meynert/ substantia innominate (NBM/SI) in young (3 month old) and aged (12 month old) Ts65Dn mice [relative to normal disomic (2N) mice], to describe the changes that occur with aging, and investigate whether these changes correspond to the attentional deficits in these mice, at either or both ages. The mice were tested on a series of automated attention tasks, followed by unbiased quantitative morphometry of BFCNs in the NBM/SI. Ts65Dn mice exhibited attentional dysfunction at both ages (relative to age-matched 2N), with greater dysfunction seen in the older animals. Morphological analysis of ChAT-immunoreactive neurons in the NBM/SI revealed that the density of these neurons in this region was significantly reduced in the trisomic mice relative to the 2N, but that aging did not result in a decrease in density, number or size of these neurons, as commonly believed. In addition the morphological analyses revealed a reduction in cell size and number for 2N mice as a function of age. As a result, the number and size of these neurons in this region tended to be greater for the aged trisomic mice relative to aged matched 2N mice. Finally, cor-

relational analyses revealed that (i) for 2N mice (both ages combined), increasing number of ChAT-immunoreactive neurons in the NBM/SI was associated with improved performance in Attention Task 2, and (ii) for older animals (genotypes combined), increased size of ChAT-immunoreactive neurons in the NBM/SI was associated with poorer performance in Attention Task 2. In sum, these findings revealed that Ts65Dn mice exhibit attentional dysfunction as early as 3 months of age, which becomes more pronounced with aging. The present study also found evidence of significantly reduced density of ChAT-immunoreactive neurons in the NBM/SI in the trisomic mice, but no age-related changes in the morphology of these neurons with aging as is commonly believed. Thus, although reduced density of these neurons may contribute to the attentional dysfunction seen in Ts65Dn mice, other neural changes likely underlie the progressive decline in attentional function with aging in these mice.

INTRODUCTION

Down syndrome (DS) is the most common known cause of intellectual disability (ID), affecting 1 in 800-1000 births. This disorder is caused by triplication of human chromosome 21(HSA21) due to nondisjunction during meiosis. In addition to ID, 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).

Attentional deficits are one of the hallmarks of DS. Researchers have found shorter and fewer periods of sustained attention in toddlers with DS (Brown et al., 2003). Furthermore, chronological studies have found attentional problems in childhood that become progressively worse into adolescence and adulthood (Brown et al., 2003; Clark and Wilson, 2003; Cornish et al., 2007; Tomporowski et al., 1990; Wilding et al., 2002). These deficits are in both selective and sustained attention. Researchers believe that the development of AD-like neuropathology during the third to fourth of decade of life in DS further contributes to attentional dysfunction (Della Sala et al., 1992; Foster, 2001; Krinsky-McHale et al., 2008; Levinoff et al., 2004; Pignatti et al., 2005). For example, DS patients who have been diagnosed with AD show impairments in selective and sustained attention (Krinsky-McHale et al., 2008) in a multi-trial cancellation task that requires subjects to selectively attend to certain objects. It has been hypothesized that the attentional dyfunction seen after the onset of AD-like neuropathology may be due, at least in part, to the atrophy and loss of BFCNs in the nucleus basalis of meyner/ substantia innominata (NBM/SI) that modulate attentional functions (Krinsky-McHale et al., 2008; Sarter and Bruno,

1997), but there is no direct evidence for this link.

Several mouse models have been developed to study the relationship between the triplication of specific genes in DS and distinct phenotypic features (Das et al., 2011; Rueda et al., 2012; Salehi et al., 2006). The most well characterized model of DS is the Ts65Dn mouse (Davisson, et al., 1990 Holtzman et al., 1996), which contains over 100 highly conserved genes that are orthologous to those on HSA21 (Mural et al., 2002; Patterson and Costa, 2005; Sturgeon and Gardiner, 2011). 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, these mice show deficiencies in developmental neurogenesis and hypocellularity (Bianchi et al., 2010a; Guidi et al. 2011), which may play a role in their observed cognitive deficits (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). Furthermore, Ts65Dn mice are born with intact BFCNs, which progressively atrophy beginning at approximately 4-6 months of age specifically within the MS (Cooper et al., 2001; Granholm et al., 2000; Holtzman et al., 1996). Similar to humans with DS, Ts65Dn mice show impairments in explicit memory and spatial mapping, subserved by BFCN projections from the medial septal nucleus (MS) to the hippocampus (Granholm et al., 2000; Hyde and Crnic, 2001; Hyde et al., 2001), as well as impaired attention and emotion regulation (Driscoll et al., 2004; Moon et al., 2010), modulated by BFCN projections from the nucleus NBM/SI to the neocortex.

Our group has previously reported attentional dysfunction in aged Ts65Dn mice (Driscoll et al., 2004; Moon et al., 2010). Similar to humans with DS who show impairments in sustained attention in adulthood (Krinsky-McHale et al., 2008), aged Ts65Dn mice tested on a series of

visual attention tasks (after the onset of AD-like neuropathology) show deficiencies in their ability to focus attention and were often "off-task" compared to age-matched wild-type [disomic (2N)] mice (Driscoll et al., 2004; Moon et al., 2010). These impairments may reflect dysfunction or atrophy of cholinergic neurons in the NBM/SI and their projections to neocortex, based on the evidence for loss of these neurons in DS (Casanova et al., 1985) and AD (Casanova et al., 1985; Mann et al., 1085; Mufson et al., 2000). However, there are no reports describing the morphology of NBM/SI cholinergic neurons in aged Ts65Dn animals, and importantly none that have attempted to relate changes in this system to the attentional dysfunction seen in these animals.

The current study was designed to characterize NBM/SI cholinergic neurons in young and aged Ts65Dn mice, to describe the changes that occur with aging and investigate whether these changes correspond to progressive attentional dysfunction in these animals as they age. This study is the first to examine attentional function in young Ts65Dn mice and ascertain whether changes in NBM/SI neurons at either age underlies the attentional dysfunction seen in these animals. We employed a 2 X 2 design, with two genotypes ([Ts65Dn mice and 2N (disomic) mice]) and two ages (3 months vs. 12 months at start of testing). We administered the same series of visual attention tasks used in two prior studies from our laboratory which demonstrated attentional dysfunction in aged Ts65Dn mice (Driscoll, et al., 2004; Moon et al., 2010). Following the completion of behavioral testing, the animals were euthanized, and the brains were immunolabeled for ChAT, followed by unbiased stereological assessments of BFCNs to test the hypothesis that aging alters BFCN morphology in the NBM/SI. Finally, correlational analyses were conducted to assess the relationship between task performance and BFCN characteristics.

METHODS

Subjects

Ts65Dn and age-matched disomic (2N) male mice were purchased from Jackson Laboratories (Bar Harbor, ME) and delivered to Weill Hall at Cornell University in Ithaca NY. Before the mice were shipped to Cornell University for behavioral testing, they were typed via quantitative polymerase chain reaction (qPCR) for the detection of the extra chromosomal segment and determination of $Pde6B^{rd1}$ homozygosity, a recessive mutation leading to retinal degeneration (Bowes et al., 1993). $Pde6B^{rd1}$ homozygous mice were excluded from the study. The young mice were received at an average age of 2 months whereas the older mice arrived at an average 11 months.

Upon arrival, the mice were housed individually in polycarbonate cages with food and water *ad libitum*. Within a week of arrival, the mice were put 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. Mice were singly housed to prevent fighting between cage-mates, which often occurs when group-housed male mice of this strain are returned to the home-cage following daily behavioral testing. A combination of daily handling, testing and the provision of items in the home-cage (i.e., plastic igloos, tubes, and plastic-gel bones, Nestlets) countered the environmental impoverishment of single animal housing. The mice were maintained on a 12:12 reversed light dark cycle under temperature-controlled conditions. 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.

Food restriction scheduling during behavioral testing

The daily food allowance was gradually reduced and then maintained when the mouse reached this target weight. For the young mice, it was necessary to implement a food restriction protocol that allowed growth, since the animals were still growing at the age (2 months of age). These animals were fed ~2g/day and changes were made as needed to keep the mice sufficiently motivated enough to complete 60-70 trials per day, while allowing them to continue gaining weight. When the young mice reached the age at which mice normally attain their full adult weight (~3 months of age), a constant target weight was maintained for the duration of testing.

All animals were fed immediately after each daily test session. This consisted of subtracting the number of calories obtained as a reward in the testing chamber (Liquefied AIN-76A purified chow; Bio-Serv, Frenchtown, NJ) from the daily allowance of lab chow (Dyets; Bethlehem, PA).

Testing Apparatus

The testing apparatus and procedures were similar to that described in our prior study (Moon et al., 2010). Briefly, all subjects were tested individually in one of eight automated Plexiglas chambers, each of which was controlled by a PC and enclosed in an insulated, sound attenuating chamber. These testing chambers were originally adapted from the nine hole operant chambers developed to asses attention in mice (Humby et al., 1999, Marston et al., 2001). The slightly curved rear wall contained five circular response ports, 1 cm in diameter, located 2cm above the floor and 5mm apart. A nose-poke into any of these 5 ports constituted a response (i.e., choice). Infrared photodiodes, positioned inside each port 0.5cm from the opening, monitored the responses to each port. Green 4MA LEDs, one embedded on the back surface of each port, provided the discriminative visual cues. One the chamber wall opposite the response port was an

alcove (15 mm wide, 2 cm above the floor) containing the dipper (ENV0302M, MED Associates, East Fairfield, VT), which dispensed the liquid reward. Access to the dipper alcove was controlled by a thin metal door activated by a motor located outside of the testing chamber. Nosepokes into the alcove were monitored by infrared photodiodes. Each of the automated events in the chamber (e.g. door opening, dipper movement, responses) were timed, controlled, and recorded by custom programs written in QBASIC. Each chamber was equipped with an exhaust system that transported air at a rate of four complete air changes per minute.

Behavioral Testing

At the onset of behavioral testing, the young animals were approximately 3 months of age and the older animals were approximately 12 months of age. Each animal was randomly assigned to one of eight chambers, with the stipulation that each chamber was balanced for each of the four treatment conditions defined by genotype and age. The animals were tested six days a week, for approximately 4-5 months. Each daily session lasted for 30 minutes or 70 trials, whichever came first. All testing was conducted by experimenters who were unaware of the genotype and age of the animals. Testing equipment was thoroughly cleaned and dried following the testing of each mouse, using Odormute (R.C. Steele Co, Brockport, NY), a detergent containing an enzyme that removes olfactory cues (e.g. pheromones).

Training

Training consisted of a series of four stages designed to familiarize the animals with the testing chambers and the sequence of responses necessary to complete a trial for the visual attention tasks. During these four stages the mice learned that the door to the dipper alcove would be

raised at the start of each trial and that a nosepoke into the dipper port, followed by a nosepoke into one of five response ports, would produce the delivery of 0.01ml of the liquid diet (i.e., reward) in the dipper alcove. 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. For a more detailed description of these stages, see Driscoll et al., 2004 and Moon et al., 2010.

Five-choice visual discrimination task

In this task, one of the five port LEDs was illuminated on each trial. The port remained illuminated until the mouse made a nosepoke into one of the ports, or until 32 seconds elapsed. The mouse was rewarded for making a nose-poke intro the illuminated (i.e., correct) port. The location of the visual cue was pseudorandomized across trials; the number of cue presentations in each port was balanced for each daily session. A 2-s delay separated trial initiation and cue onset. This delay, termed the "turn-around time", allowed time for the mouse to turn around and orient toward the ports before cue illumination. Four types of errors were distinguished: (1) a nose-poke into any response port prior to cue onset was termed a premature response; (2) a nosepoke made following cue onset but to one of the non-illuminated ports was termed an innacurate response; and (3) failing to respond to any response port within 5sec after the cue (omission error). A 5-second intertrial interval (ITI) separated adjacent trials. All trials in which the mouse made an initiation poke into the dipper alcove (regradless of the outcome of the trial) were defined as response trials. Failures to initiate a trial within 60 seconds were scored as "nontrials; no cues were presented on these trials. A 5-second time-out period was imposed following a nontrial or an error. This time-out period was signaled by the illumination of a 3-W

houselight on the ceiling of the chamber. Each mouse remained on this task until it reached a criterion of 80% correct for 2 out of 3 consecutive sessions.

Attention Tasks 1 and 2

The mice were subsequently tested on two variants of the initial visual discrimination task that were identical except for the duration of the cue illumination, which was shortened to 2 seconds and 1 second, respectively, for the two tasks. These tasks tested attentional function and prepared the mice for the subsequent two attentional tasks, which were more attentionally-demanding. Attention tasks 1 and 2 were administered for 8 and 15 sessions, respectively.

Attention Task 3 (learning to wait for the cue)

This task was identical to the prior task except that the duration between trial initiation and cue onset varied pseudo-randomly across trials. The pre-cue delay varied between 0, 2, 4 seconds, all added to the 2-second "turn around time" (described above). Cue illumination duration was constant at 1 second. If a response was made prior to cue onset (i.e., premature response), the trial was terminated and no cue was presented. As noted above, premature responses (as well as all types of errors) were followed by a 5-second time-out period, signaled by the illumination of a 3-W houselight on the ceiling of the chamber. The three pre-cue delays were presented pseudo-randomly, such that the number of presentations of each combination of pre-cue delay and response port (1-5) was balanced across each session. The mice were tested for 18 sessions on this task. The early sessions on this task tap the ability of the mice to learn to wait for the cue, whereas the later sessions provide a more pure index of inhibitory control and focused attention. Thus, this task taps learning, inhibitory control, focused attention.

Tissue preparation

Upon completion of behavioral testing, mice were deeply anesthetized with ketamine (85 mg/kg)/xylazine (13 mg/kg) via intraperitoneal injection, perfused transcardially with 0.9% saline (50 ml), followed by 4% paraformaldehyde (50 ml) in phosphate buffer (PB; 0.1M; pH = 7.4). Ages at sacrifice averaged 7.6 months for the young mice and 16.4 for older mice. Brains were extracted from the calvaria, post-fixed for 24 h in the same fixative, and placed in a 30% sucrose PB solution at 4 °C until sectioning. Each brain was cut in the coronal plane at 40 µm thickness, on a sliding freezing microtome into six adjacent series and stored at 4 °C in a cryoprotectant solution (30 % ethylene glycol, 30 % glycerol, in 0.1 M PB) prior to immunohistochemical staining (Ash et al., 2011; Kelley et al., 2014a, 2014b; Velazquez et al., 2013).

Immunohistochemistry

Immunohistochemistry was performed as previously described (Kelley et al., 2014a, 2014b). Tissue was immunostained using the primary antibody: a goat polyclonal antibody against choline acetyltransferase (ChAT, 1:1000; Millipore, Billerica, MA). A 1/6 series of sections was singly immunolabeled for ChAT. Briefly, sections were washed in 0.1 M phosphate buffer (PB; pH 7.4) to remove cryoprotectant, rinsed in Tris-buffered saline (TBS), and incubated in sodium (meta)periodate (2.139 g per 100 ml TBS) to inhibit endogenous peroxidase activity. To improve primary antibody penetrance throughout the full depth of the tissue, sections was washed in TBS with 0.25 % Triton X-100, followed by rinses in a blocking solution to prevent nonspecific binding consisting of 3 % serum (raised against host organism of secondary antibody: ChAT, horse serum) in TBS/Triton X-100. Tissue was then incubated overnight at room temperature with the primary antibody in TBS/Triton X-100 with 1 % serum and then washed in TBS

and incubated for 1 h with a biotinylated IgG secondary antibody raised against the host of the primary antibody, ChAT anti-goat (Vector Laboratories, Burlingame, CA). Tissue was washed in TBS and incubated with an avidin-biotin complex (ABC) solution (Elite Kit, Vector Laboratories) for 1 h to amplify the immunochemical reaction. Immunolabeling was visualized using an acetate-imidazole buffer containing 0.05 % 3,3´-diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich, St. Louis, MO) and 0.0015 % freshly prepared H_2O_2. Sections were washed in acetate-imidazole buffer to terminate the immunochemical reaction, mounted onto chrome-alum-subbed slides, air dried for 24 h, dehydrated through a series of graded alcohols (70%, 95%, and 100 %), cleared in xylenes, and cover-slipped with distyrene/dibutylphthalate (plasticizer)/xylene (DPX) mounting medium (Kelley et al., 2014a, 2014b; Velazquez et al., 2013).

Stereology

ChAT -immunoreactive neuron counts were determined in the NBM/SI using the optical fractionator, a stereological model 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, USA) coupled to a Nikon Optiphot-2 microscope. 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 x 50 µm counting frame, 151 x 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 frac-

tion allowed for a $CE_{m=1}$ of ≤ 0.10 (Gundersen, 1999). Cell density is presented as cells per 1,000,000 μm^3 (Kelley et al., 2014a, 2014b). Calculation was performed for each animal, prior to group averages. Photomicrographs were taken on a Nikon Optiphot-2 microscope (Tokyo, Japan) connected to Stereo Investigator software (MicroBrightField, Inc.) Background correction was used at the time of image capture to establish evenness of illumination across the field, and scale bars were added within the Stereo Investigator software. Panels were compiled in PowerPoint (version 14.0.6129.5000, 32-bit, Microsoft, Redmond, WA, USA) and each micrograph was equally corrected for brightness and contrast. No retouching or further manipulations were performed.

Neuron size

BFCN size 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).

Antibody tissue penetration

A major criterion for the use of the optical disector is antibody penetration through the full depth of the stained section. The depth of ChAT antibody penetration through a tissue section in the z-axis was determined using the same optical disector system and software used to

count labeled neurons in this study (see above). ChAT antibodies penetrated the full depth of the section allowing for the equal probability of counting all objects, a prerequisite for stereology (Mufson et al., 2000; Kelley et al., 2014a, 2014b; Velazquez et al., 2013).

Statistical Analyses

Statistical analyses were performed using the Statistical Analysis System ***(***Version 9.1; SAS Institute, Cary, NC***).*** Performance measures were analyzed using PROC GLIMMIX program, a generalized linear mixed model procedure for conducting repeated measures analyses of both normal and non-normal data by specifying an appropriate link function and error distribution (Wolfinger et al., 1993). The dependent measures included: percent correct, percent omissions errors, percent inaccurate responses and percent premature responses. If a violation of homogeneity of variance was present [determined by the Levene test of homogeneity (Levene, 1960)], a non-parametric analysis (i.e., Kruskall-Wallis test) was utilized.

The primary dependent measures for the morphological data were the (1) number, (2) density, and (3) volume of ChAT-immunoreative cells within the NBM/SI. These analsyes were conducted using 2X2 ANOVAs.

Lastly, Spearman's rank correlation coefficients were used to assess the relationships between percent correct in attention task 2 and number, density and volume of ChAT-positive cells within the NBM/SI. Attention task 2 was selected for these correlation analyses as it revealed progressive attentional dysfunction in the trisomic mice as a function of age, providing an opportunity to investigate the underlying neural basis of this effect.

The alpha level was set at 5% for primary analyses of both behavioral and morphological endpoints. However, a Bonferroni-Holm step down procedure was used to control for multiple

comparisons, as four *a-priori* contrasts were conducted regardless of whether or not the interaction of genotype and age was significant, because they are central to the goals of the study; these four comparisons were: (1) disomic mice who started testing at 3 months of age (2N-Y) vs. trisomic mice who started testing at 3 months of age (Ts56Dn-Y), (2) Ts65Dn-Y vs. Ts65Dn-O (trisomic mice who started testing at 12 months of age), (3) 2N-Y vs. 2N-O (disomic mice who started testing at 12 months of age) and (4) 2N-O vs. Ts65Dn-O. Using the Bonferroni-Holm step down procedure, the criteria for significance for these tests were: 0.05/4 ($P = 0.0125$) for comparison 1, 0.05/3 ($P = 0.0167$ for comparison 2, 0.05/2 ($P = 0.025$) for comparison 3, and 0.05/1 (P = 0.05) for comparison 4.

RESULTS

Final sample size

The final sample size for all behavioral and morphometric measures is presented in the figures for each task. Differences in sample size between the various tasks and the morphological endpoints resulted from several factors, including deletion of data due to apparatus malfunction, experimenter error, and tissue labeling variability.

Body Weight

Analysis of body weight at the completion of behavioral testing revealed significant effects of genotype ($F_{(1, 34)} = 16.12$, $P = 0.0003$) and age ($F_{(1, 34)} = 18.31$, $P = 0.0001$); the interaction failed to achieve statistical significance ($F_{(1, 34)} = 3.52$, $P = 0.07$). Ts65Dn mice (mean = 21.03 g; S.E.M. = 0.63) weighed significantly less than 2N mice (mean = 23.6 g; S.E.M. = 0.63). Furthermore, younger mice (mean = 20.95 g; S.E.M. = 0.59) weighed less than older mice (mean

= 23.6 g; S.E.M. = 0.66). This effect of the trisomy on body weight is consistent with prior reports (Ash et al., 2011; Bianchi et al., 2010a; Fuchs et al., 2012; Roper et al, 2012; Velazquez et al., 2013).

Five-choice visual discrimination task (no pre-cue delay, cue duration: 32s)

The dependent measure for learning rate in the initial visual discrimination task – sessions to criterion – was analyzed using the nonparametric Kruskal-Wallis test, because the data violated the assumption of homogeneity of variance. This test revealed a significant effect of group ($H_{(3)} = 9.857$, $P = 0.0198$; Fig. 1A). Subsequent post-hoc tests revealed a significant effect of aging for the 2N mice; i.e., 2N-O mice reached criterion significantly more slowly than the 2N-Y group ($P = 0.0027$). In addition, the trisomy tended to impair performance of the younger animals ($P = 0.0416$), although this comparison did not meet significance for the Bonferroni-holm correction. In contrast, for the older animals, learning rate was not affected by the trisomy.

Attention Task 1 (no pre-cue delay, cue duration: 2s, 8 sessions)

The analysis of percentage correct revealed a significant main effect of genotype ($F_{(1, 34)} = 15.79$, $P = 0.0003$; Fig. 1B): Ts65Dn mice performed significantly more poorly than their 2N counterparts. There was also a main effect of age ($F_{(1, 34)} = 10.50$, $P = 0.003$) reflecting the fact that the older mice performed significantly worse than younger animals. There was no significant genotype x age interaction ($F_{(1, 34)} = 1.33$, $P = 0.257$) suggesting that the magnitude of the impairment of the trisomic mice (relative to 2N) was comparable at the two ages. Planned contrasts revealed that the Ts65Dn-Y mice were significantly impaired relative to the 2N-Y mice ($P =$

0.0005). 2N-O animals were also impaired relative to the 2N-Y mice ($P = 0.0040$). No other significant comparisons were found.

Percent omission errors were analyzed using the nonparametric Kruskal-Wallis test because the data violated the assumption of homogeneity of variance. This test revealed a significant effect of group ($H_{(3)} = 8.54$, $P = 0.031$). Subsequent post-hoc tests revealed a borderline significant effect of aging for the 2N mice; i.e., 2N-Y mice committed a lower percentage of omission errors then the 2N-O group ($P = 0.053$, Fig. 1C), however this comparisons did not meet significance for the Bonferonni-holm correction. There were no other significant comparisons.

The analysis of percent inaccurate responses revealed a significant effect of age ($F_{(1,34)} = 4.58$, $P = 0.04$), reflecting the fact that the older mice committed a significantly higher percentage of inaccurate responses than the younger animals. Neither the main effect of genotype ($F_{(1, 34)} = 3.28$, $P = 0.08$) nor the interaction of genotype x age ($F_{(1, 34)} = 2.48$, $P = 0.124$) achieved statistical significance. Planned comparisons revealed that within the young mice, a genotype effect was seen, with the trisomics committing a higher percentage of inaccurate responses than their 2N counterparts ($P = 0.0161$, Fig. 1D). The old 2N mice had a significantly higher percentage of inaccurate responses than their younger counterparts ($P = 0.0133$). No other contrasts were significant.

Attention Task 2 (no pre-cue delay, cue duration: 1s, 15 sessions)

The analysis of percentage correct revealed significant main effects of genotype (*F*

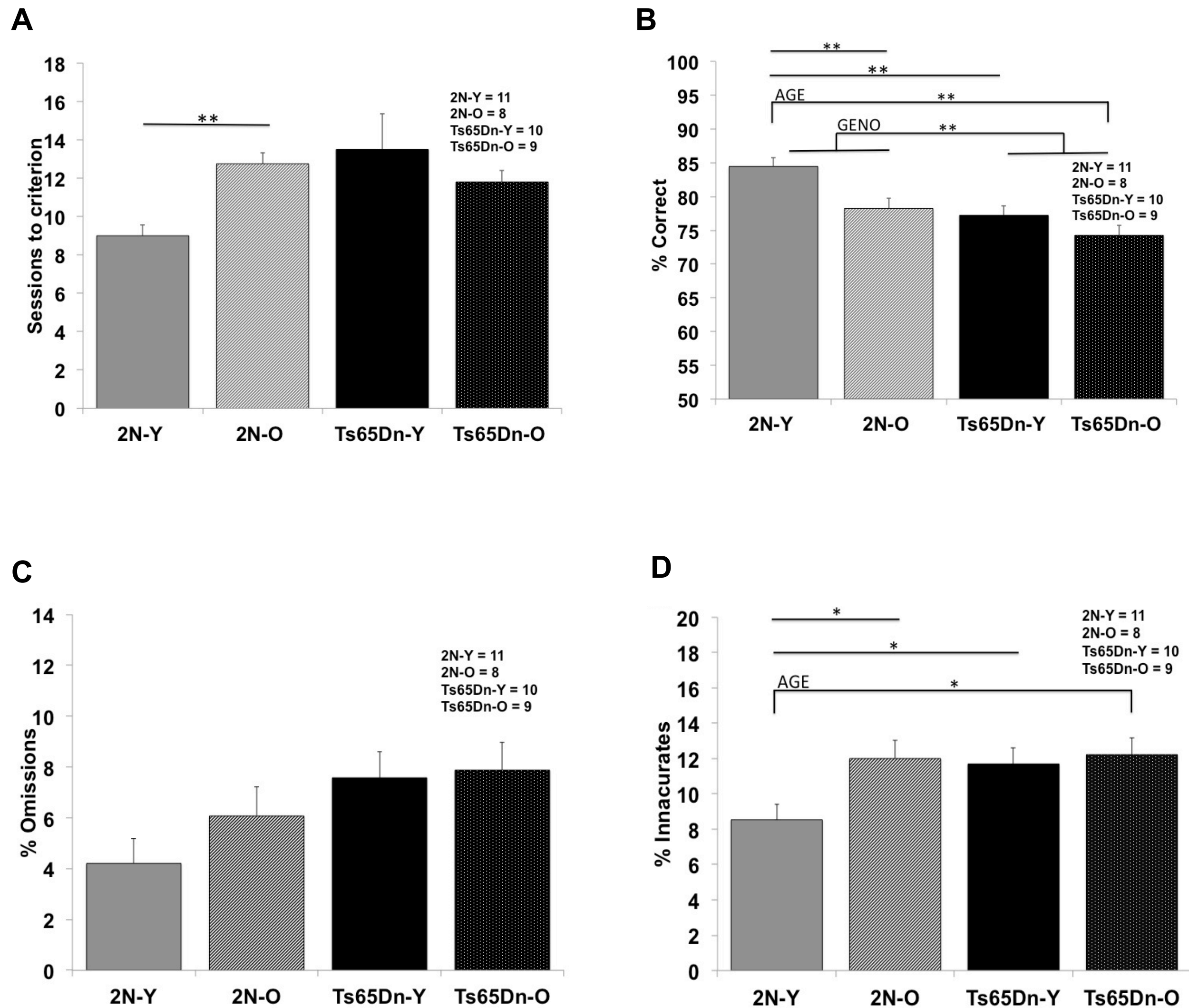

Figure. 1 Performance in the visual discrimination task (panel A) and Attention Task 1 (Panels B-D). (A) Mean (±SEM) sessions to criterion in the initial visual discrimination task. 2N-Y mice reached criterion significantly faster then the 2N-O group (*p* = .0027). **(B) Mean (±SEM) percent correct in attention task 1.** A significant main effect of genotype confirmed a performance deficit in Ts65Dn mice (*p* = .0003). A significant main effect of age indicated better performance in young mice (*p* = .003). Planned contrasts revealed significant impairment in Ts65Dn-Y mice compared to young 2N (p = .0005), and a detrimental effect of aging in the 2N mice (p = .0040). (C) **Mean (±SEM) omission errors during attention task 1.** A borderline significant contrast reveals that 2N-Y mice tended to commit a lower percentage of omission errors than the 2N-O group (*p* = .053). (D) **Mean (±SEM) percentage of inaccurate responses during attention task 1.** A significant main effect of age indicated a higher percentage of inaccurate response for the older mice (*p* = .003). Planned contrasts revealed an effect of genotype in the young mice (*p* = .0161) and an effect of aging for the 2N mice (*p* = .0133)* $p \leq 0.05$, ** $p \leq 0.01$.

$_{(1,37.29)}$ = 12.68, P = 0.001) and age ($F_{(1,37.29)}$ = 9.48, P = 0.004; Fig. 2A), as well as a significant interaction of these two variables ($F_{(1,37.29)}$ = 4.09, P = 0.05). Post-hoc analyses revealed that whereas there was no effect of age in the 2N mice, the old Ts65Dn mice performed significantly less well than their younger counterparts (P = 0.0008). In addition, although the younger trisomic mice did not perform more poorly than their 2N counterparts, the old trisomic mice were significantly impaired relative to the old 2N (P = 0.005).

The analysis of percentage omission errors revealed a significant main effect of genotype ($F_{(1,32)}$ = 33.69, P < 0.0001) as well as a significant genotype x age interaction ($F_{(1,32)}$ = 6.01, P = 0.02; Fig. 2B). Post-hoc analyses revealed a significant effect of age for this measure in the trisomic mice (P = 0.0075), but not the 2N. In addition, the trisomy increased the percentage of omission errors in the older mice (P < 0.0001), but not in the younger mice. The effect of age was not significant ($F_{(1,32)}$ = 2.51, P = 0.126).

The analysis of percentage inaccurate responses did not reveal significant effects of age, genotype, nor the interaction of these two variables.

Attention Task 3 (Variable pre cue delay: 0, 2, 4 s; constant 1 s cue duration; 18 sessions)

The analysis of percentage correct revealed a significant effect of pre-cue delay ($F_{(2, 302.7)}$ = 605.07, P < 0.0001), reflecting the fact that performance declined as pre-cue delay increased. Session-block (6 blocks of 3 sessions each) was also significant ($F_{(5, 208.8)}$ = 154.73, P < 0.0001), indicating the pronounced improvement in performance with continued training. A main effect of genotype was seen ($F_{(1, 40.21)}$ = 5.30, P = 0.03) as well as a significant genotype x delay x session block interaction ($F_{(10, 300.7)}$ = 3.71, P = 0.0001, Fig. 3). Contrasts revealed that for trials

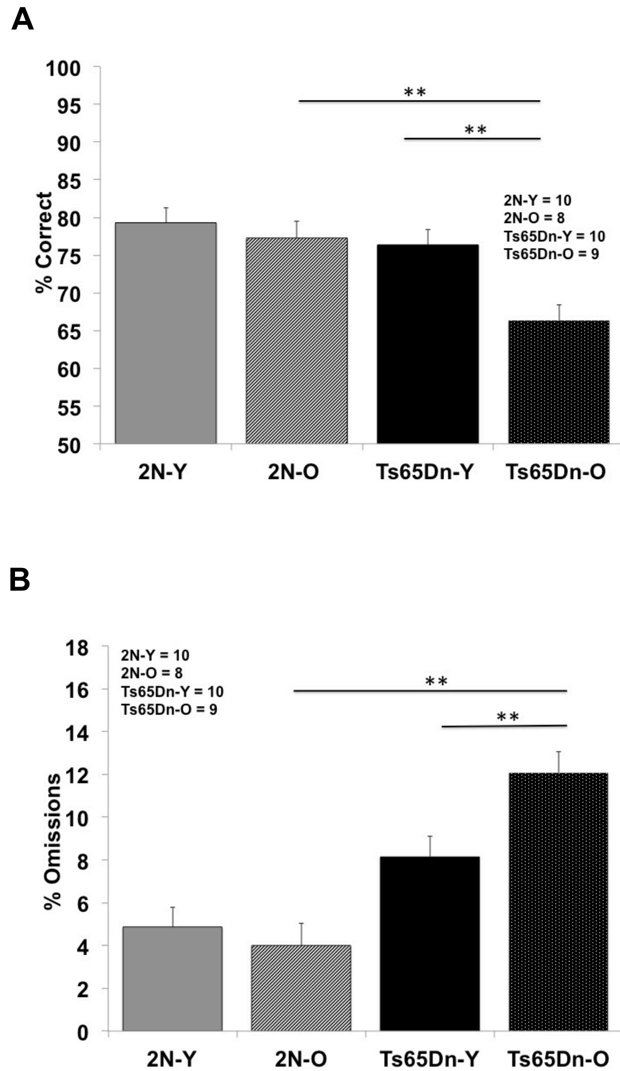

Figure 2. Performance in Attention Task 2. (A) Mean (±SEM) percent correct. A genotype x age interaction revealed that for the older mice, the Ts65Dn mice performed more poorly than their 2N counterparts (*p* = .005), whereas no genotype effect was seen for the young mice. Contrasts also revealed a significant effect of aging in the Ts65Dn mice (*p* = .0008) but not the 2N. **(B) Mean (±SEM) percent omission errors.** Similarly, a significant genotype x age interaction revealed a significant effect of the trisomy for the old mice (p <. 0001) but not the young mice, and a significant effect of aging for the Ts65Dn mice (*p* = .0075), but not the 2N. * $p \leq 0.05$, ** $p \leq 0.01$.

with a 0 sec pre-cue delay, a significant geno type effect was seen during block 1, reflecting inferior performance of the Ts65Dn mice ($P = 0.003$, Fig. 3A). For trials with a 2sec pre-cue delay, Ts65Dn mice performed worse than 2N mice during blocks 3 ($P = 0.0186$, Fig. 3C), 4 ($P = 0.003$, Fig. 3B), and 5 ($P = 0.0661$), although only the session-block 4 contrast was significant after the Bonferroni-Holm correction. Lastly, for trials with a 4sec pre-cue delay, Ts65Dn mice performed significantly better than 2N mice during block 1 ($P = 0.0124$), but worse than the 2N during blocks 4 ($P = 0.0037$) and 5 ($P < 0.0001$).

A three -way interaction of genotype, age, and pre-cue delay also found ($F_{(2, 302.7)} = 3.42$, $P = 0.034$). This three way interaction was driven by that fact that whereas the pattern of effects for the two young groups was constant across delay (inferior performance of the old animals), the pattern for the older animals tended to vary across delay. However, because the two old groups did not differ significantly at any delay, coupled with the clear importance of session-block (described above) in describing the influence of genotype and delay, it was deemed preferable to discuss the three way interaction of genotype, delay and session-block.

The analysis of percentage premature responses revealed a highly significant effect of pre-cue delay ($F_{(1, 223.6)} = 457.64$, $P < 0.0001$), reflecting the fact that the percentage of premature responses increased as the interval prior to cue presentation increased. Session-block (6 blocks of 3 sessions each) was also highly significant ($F_{(5, 121.5)} = 82.96$, $P < 0.0001$), indicating a decrease in the percentage of premature responses with continued training, as the animals learned to wait for the cue. There was also a genotype x session-block interaction ($F_{(5, 121.5)} = 4.23$, $P = 0.001$) revealing that 2N mice committed a higher percentage of premature responses than Ts65Dn mice during the first block only ($P = .0019$, Fig. 4A). The genotypes did not differ in the percentage of premature responses throughout the remaining sessions blocks.

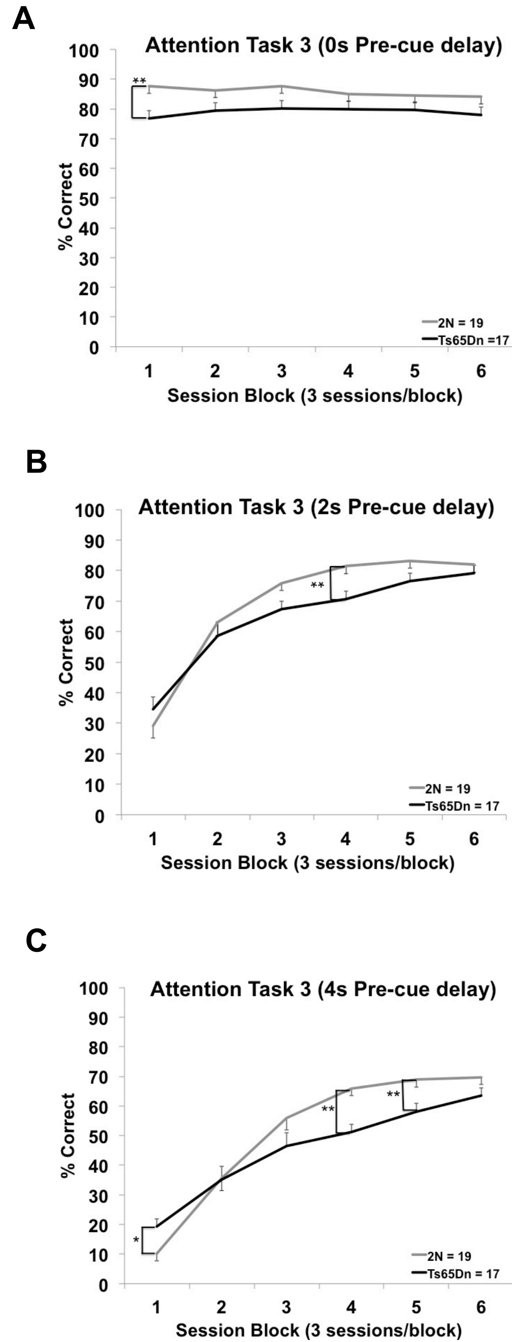

Figure 3. Mean percentage correct (±SEM) in Attention task 3, for each of the three pre-cue delays. **(A)** For trials with a 0sec pre-cue delay, no effect of genotype was seen except for block 1 (p =. 003). **(B)** For trials with a 2sec pre-cue delay, Ts65Dn mice performed significantly worse than 2N mice in block 4 only (p =. 003). **(C)** For trials with a 4sec pre-cue delay, the trisomics performed worse than the 2N during blocks 4 (p = .0037) and 5 (< .0001), whereas the opposite pattern was seen for block 1 (p = .0124). $p \leq 0.05$, ** $p \leq 0.01$.

The analysis of percentage omission errors revealed a significant effect of pre-cue delay ($F_{(2, 86.24)} = 7.91$, $P = 0.0007$), reflecting the fact that the percentage of omission responses increased as the interval prior to cue presentation increased. A highly significant effect of genotype was seen ($F_{(1, 19.49)} = 18.98$, $P = 0.0003$, Fig. 4B), reflecting that the trisomics committed a significantly higher percentage of omission errors than the 2N mice in this task. There was no significant main effect of age for percent omission errors ($F_{(1, 19.49} = 0.02$, $P = 0.8887$) nor significant interactions involving age.

Lastly, the analysis of percentage inaccurate responses revealed a main effect of age ($F_{(1, 32.07)} = 4.54$, $P = 0.0408$) as well as a genotype x age interaction ($F_{(1, 32.07)} = 4.06$ $P = 0.05$, Fig. 4C). Post-hoc analyzes revealed a significant effect of age for this measure in the 2N mice but not for the trisomics; 2N-O had a higher percentage of inaccurate responses than the 2N-Y ($P = 0.0052$).

Morphology of ChAT-immunoreactive cells in the NBM/SI

ChAT-immunoreactive cell number

The analysis of ChAT-immunoreactive cell count in the NBM/SI did not reveal a main effect of genotype ($F_{(1, 55)} = 0.80$, $P = 0.3762$, Fig. 5A) nor a genotype x age interaction ($F_{(1, 55)} = 1.44$, $P = 0.2356$). There was a significant main effect of age ($F_{(1, 55)} = 6.53$, $P = 0.0134$), revealing a 16.6% decrease in in ChAT-immunoreactive cell number in older animals compared to their younger counterparts. Planned contrasts revealed a significant reduction in ChAT-immunoreactive cell number in the 2N-O mice relative to their younger counterparts ($P = 0.0086$, Fig. 5A). The remaining contrasts failed to reach significance.

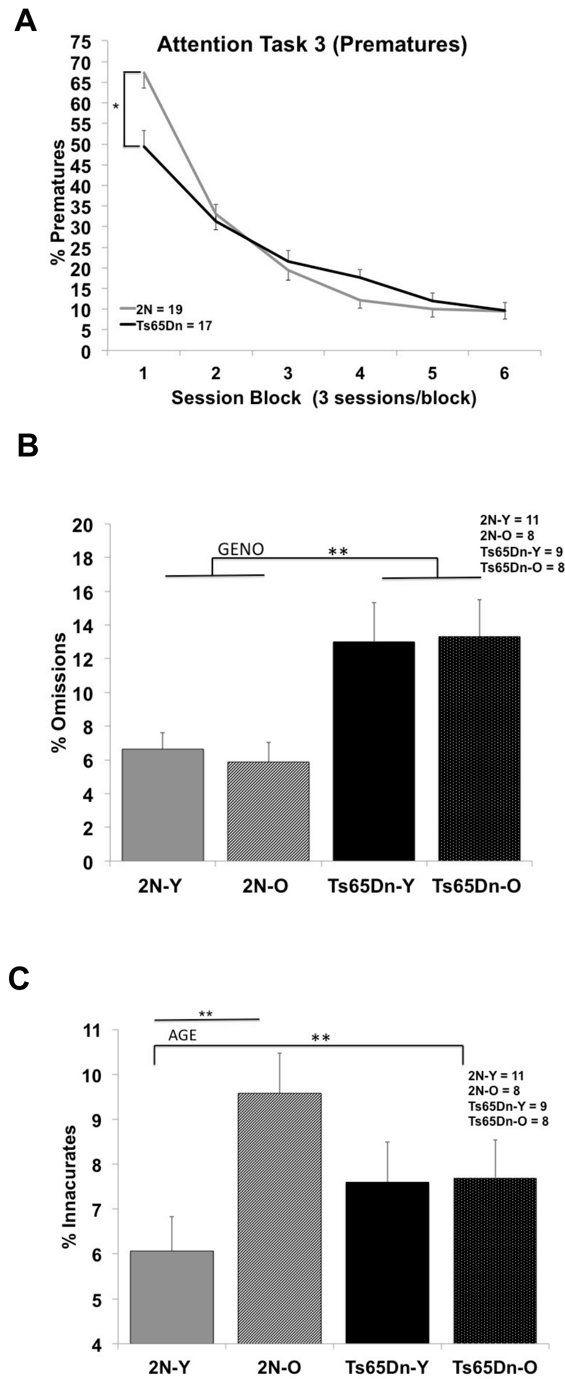

Figure 4. Performance on Attention Task 3. (A) Mean (±SEM) premature responses, as a function of session-block. A significant genotype x session-block interaction revealed that genotype differences were seen only during block 1, where the 2N mice committed a higher percentage of premature responses than Ts65Dn mice (p = .0019). **(B) Mean (±SEM) omission errors.** Ts65Dn mice committed a higher percentage of omission errors than the 2N mice (P = 0.0003). (C) **Mean (±SEM) percentage of inaccurate responses** A significant genotype x age interaction revealed that aging increased the percentage of inaccurate responses for 2N mice (p = .0052) but not trisomics. $p \leq 0.05$, ** $p \leq 0.01$.

ChAT-immunoreactive cell density

Genotype significantly altered ChAT-immunoreactive neuron density within the NBM/SI ($F_{(1, 55)} = 3.99$, $P = 0.05$, Fig. 5B): Ts65Dn mice showed a 9.72% reduction relative to the 2N group. The effect of age was not significant ($F_{(1, 55)} = 1.31$, $P = 0.2568$,) nor was the interaction of genotype and age ($F_{(1, 55)} = 0.36$, $P = 0.5523$). Planned contrasts revealed a borderline significant reduction in density for the Ts65Dn-Y animals relative to the 2N-Y ($P = 0.0697$), however this comparison did not meet significance for the Bonferonni-holm correction. All other comparisons failed to reach significance.

ChAT-immunoreactive cell size

The analysis of ChAT-immunoreactive neuron size within the NBM/SI revealed a significant main effect of genotype ($F_{(1, 55)} = 4.48$, $P = 0.0321$) and a borderline effect of age ($F_{(1, 55)} = 3.99$, $P = 0.0508$). There was also a significant genotype x age interaction ($F_{(1, 55)} = 4.87$, $P = 0.0316$, Fig. 5C). Contrasts revealed that for the 2N mice, advancing age resulted in atrophy of ChAT-immunoreactive neurons ($P = 0.0035$), whereas no effect of age was seen for the Ts65Dn mice. Accordingly, a significant genotype effect was seen for the older mice, whereby, the older 2N mice exhibited significantly smaller ChAT-immunoreactive cells than the older trisomic mice ($P = 0.0031$).

Correlations between percent correct in Task 2 and morphology of ChAT immunoreactive cells in the NBM/SI

Some measures of NBM/SI BFCN morphology significantly predicted percentage correct in Task 2. For the older mice, a significant negative correlation was seen between percent

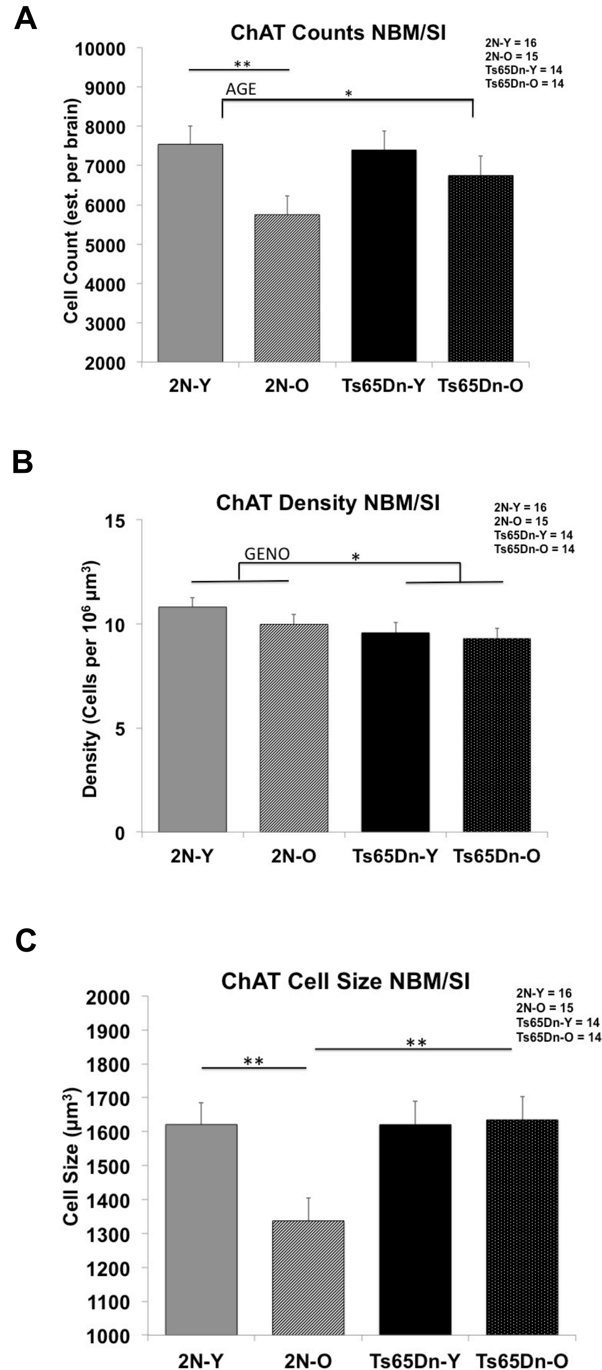

Figure 5. Morphology of ChAT immunoreactive neurons in the NB/SI (A) Mean (±SEM) cell count. A main effect of age indicated that **o**lder mice exhibited fewer cells than younger mice (*p* =. 0134). **(B) Mean (±SEM) cell density.** A significant main effect of genotype indicated reduced density in the tri-somics (*p* = .05). **(C) Mean (±SEM) cell size.** A significant interaction of genotype x age reflected the fact that age decreased neuron size for the 2N (*p* = .0035) but not the trisomic mice. Accordingly, the older 2N mice had smaller ChAT-immunoreactive cells than the older trisomic mice (*P* = 0.0031). $*p \leq 0.05$, $** p \leq 0.01$.

correct and ChAT-immunoreactive cell size in the NBM/SI (r_s = -.5412, P = 0.046 Fig. 6A), reflecting a decrease in performance with increasing neuron size. In addition, for the 2N mice, a borderline significant positive association was seen between percent correct and ChAT-immuno-reactive cell number within the NBM/SI (r_s = .4823, P = 0.058 Fig. 6B); i.e., as neuron number increased, performance improved.

DISCUSSION

The results of the current study demonstrated impairments in learning and attention in the trisomic mice, recapitulating hallmark areas of dysfunction in children and adults with DS (Brown et al., 2003; Cornish et al., 2007; Clark and Wilson, 2003; Krinsky-McHale et al., 2008; Wilding and Cornish, 2004). Importantly, this study is the first to characterize the effects of aging on either attentional function or morphology of NBM/SI neurons in Ts65Dn mice. The assessment of both endpoints within the same animals provided a unique opportunity to assess functional relationships.

Comparison of the young 2N and trisomic mice

The young trisomic mice tended to learn the initial visual discrimination task more slowly than their 2N counterparts (see Fig. 1A). The fact that the young Ts65Dn mice eventually reached a high level of performance on this task indicates that their slower learning in this task was not due to deficits in visual acuity, motor function, or motivation but rather appears to reflect impaired associative learning, as reported for children and adults with DS (Ohlrich and Ross, 1968; Woodruff-Pak et al. 1996).

This series of tasks also provided new evidence of attentional dysfunction in young

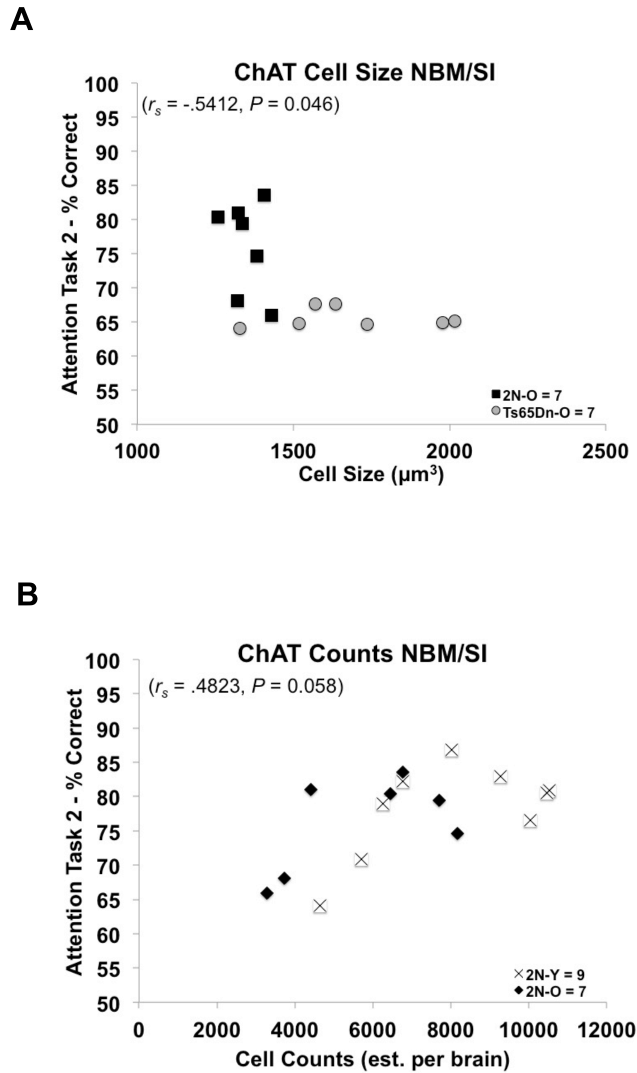

Figure 6. Correlations between performance in Attention Task 2 and morphological characteristics of ChAT-immunoreactive neurons in the NB/SI. (A) For the old mice (both genotypes combined), a significant negative correlation was seen between percent correct and ChAT-immunoreactive cell size (r_s = -.5412, P = 0.046); i.e. as ChAT-immunoreactive cell size increased, percent correct decreased. **(B)** 2N mice (old and young combined) showed a borderline significant positive correlation between percent correct and ChAT-immunoreactive cell number within the NBM/SI (r_s = .4823, P = 0.058); i.e., as neuron number increased, performance improved.

Ts65Dn mice. They committed a significantly higher percentage of inaccurate responses in attention task 1, the first task in which cue duration was relatively brief, thereby increasing attentional demands. Since the rules of the task had already been mastered prior to this task, the most ikely explanation for the increased incidence of inaccurate responses by the young trisomic mice (v 2N-Y) is attentional dysfunction; i.e., missing the cue due to lapses in attention and then guessing between two ports in the general vicinity of the illuminated port which is consistent with prior reports (Driscoll et al., 2004). This inference is supported by the types of errors committed in attention Task 3, the first task that included variable delays before cue presentation. In this latter task, the Ts65Dn-Y mice performed more poorly than the young 2N mice, and importantly, the magnitude of the impairment appeared to become progressively more pronounced as the delay before cue onset increased (Fig 3A-C). Analysis of the various types of errors revealed that this impairment in performance (percentage correct) was driven primarily by an increased incidence of omission errors by the trisomics (Fig. 4B), indicative of missing the cue. Ts65Dn mice did not commit a higher percentage of premature responses than the 2N animals, indicating that their impairment for trials with longer pre-cue delays was not due to impaired inhibitory control but rather an impaired ability to maintain attentional focus until cue presentation. In sum, these finding suggest that young TS65Dn mice are impaired relative to young 2N mice in tasks where they must wait for a cue and maintain focused attention during the delay. These findings are consistent with the human literature showing that children and young adults with DS are impaired in sustained attention (Brown et al., 2003; Cornish et al., 2007; Tomporowski et al., 1990; Wilding et al., 2002).

Attention in aged Ts65Dn mice (vs. aged 2N mice)

Old Ts65Dn mice did not differ from their 2N counterparts in rate of learning the initial visual discrimination task, consistent with previous findings (Driscoll et al., 2004, Moon et al., 2010; Powers et al., in preparation). When the visual cue duration was shortened to 2sec in attention task 1, there were no detectable differences between old trisomics and 2N mice. However, when the cue duration was made even briefer in attention task 2 (1 sec), Ts65Dn-O animals performed significantly worse than their 2N counterparts (Fig. 2A), consistent with our prior findings (Driscoll et al., 2004; Moon et al., 2010; Powers et al., in preparation). This impairment was driven by an increased percentage of omission errors (Fig. 2B), suggesting an inability of the trisomics to maintain attentional focus. When further challenging the mice with a variable pre-cue delay in attention task 3, the trisomics, regardless of age, were impaired relative to the 2N mice (Fig. 3B, C), with the magnitude of the impairment appearing to get larger with increasing pre-cue delay. This impairment in percent correct was driven by an increased incidence of omissions by the trisomic mice (Fig. 4B), further indicating that this deficit was due to impaired attention, not impaired inhibitory control, consistent with prior reports (Driscoll et al., 2004, Moon et al., 2010; Powers et al., in preparation). This inference is further supported by the results of videotape analyses presented in a prior report from our laboratory. These analyses revealed that the aged trisomics were frequently off-task, exploring in the side of the chamber opposite to the ports. This behavior may indicate dementia due to AD-like pathology, in addition to impaired sustained attention (Driscoll et al., 2004). In sum, these finding suggest that aged TS65Dn mice are impaired relative to age-matched 2N mice in tasks which require focused attention.

Comparison of the young and old Ts65Dn mice

It was hypothesized that attentional dysfunction of the trisomic mice would become more pronounced with aging due to atrophy of BFCNs, particularly those of the NBM/SI. This proved to be the case in some, but not all tasks. The old Ts65Dn mice did not differ from their young trisomics mice in performance of attention task 1, the first task in which a relatively brief cue was used. However, when cue duration was further decreased to 1s duration, placing even greater demands on attention, the older trisomics did, in fact, perform significantly more poorly than the young trisomics. This decreased percent correct seen in older trisomics (relative to their younger counterparts) was driven by an increased percentage of omission errors (Fig. 2B), suggesting impaired focused attention in the older trisomic mice, or a greater incidence of being off task, as discussed above (Driscoll et al., 2004). In attention task 3, as discussed previously, both young and old trisomics were impaired when compared to 2N animals, driven primarily by an increased incidence of omission errors. However, in this task the two trisomic groups did not differ from each other. In sum, these finding suggest that older TS65Dn mice are impaired relative to younger trisomics in tasks which require focused attention but predictable cue onset, whereas these groups are both impaired, but to a similar degree, in tasks requiring sustaining attention over a variable delay.

Effects of aging in the 2N mice

A growing body of evidence suggests that normal aging results in loss of attentional function in humans (Parasuraman and Giambra 1991; Greenwood et al., 1997; Berardi et al., 2001) and in laboratory rodents (Jones et al. 1995; McGaughy and Sarter 1995; Muir et al.1999). The present study revealed impairments in both learning and attention in the normal 2N mice as

a function of age. First, a clear effect of aging was seen in the rate at which the 2N mice learned the initial visual discrimination task. Younger 2N mice reached criterion significantly fasten then the older 2N counterparts (Fig. 1A). This finding is consistent with a prior report, which found that 3month old rats are faster at learning the initial rules of the 5-choice serial reaction task of attention than 15month old animals (5-CSRT; Jones et al., 1995).

A detrimental effect of aging was also seen for the 2N mice in attention task 1, when the cue duration was made relatively brief (2 s) but still with a predictable onset time (Fig. 1B). This performance deficit was driven by increased percentage of omission errors by the older 2N mice, indicative of missing the cue. In addition, in Attention Task 3, the older 2N mice committed a significantly higher percentage of inaccurate responses also indicative of a deficit in maintaining attentional focus. This pattern of findings indicates that aging produces impairments in both learning and attention in normal disomic mice, as seen in humans (Parasuraman and Giambra 1991; Greenwood et al., 1997; Berardi et al., 2001).

Assessment of BFCNs and their relationship to cognitive function

We utilized quantitative stereology to obtain unbiased measures of ChAT-immunolabled BFCNs in the NBM/SI. BFCNs in the NBM/SI project to neocortex and are important in modulating attention (Hasselmo and Sarter, 2011; Mesulam et al., 1983; Rye et al., 1984). Although there has been work investigating BFCNs in Ts65Dn, most prior research has focused on the BFCNs in the MS in older animals. This study is the first to examine BFCN morphology in the NBM/SI of behaviorally tested animals at young and old ages.

A main effect of age revealed a 16.6% reduction in the number of ChAT-immunolabeled neurons within the NBM/SI (Fig. 5A) of older mice. Planned contrasts

revealed that this effect of age was more pronounced for the 2N mice than the trisomics. For density of these neurons within the NBM/SI, a genotype effect revealed a 9.72% decrease within the NBM/SI for trisomics relative to 2N mice, with a comparable effect for the two age groups. The reduced density of these neurons may contribute to the attentional dysfunction seen in Ts65Dn mice; however, because the magnitude of this effect was comparable for the two age groups, it seems likely that other neural changes may underlie the progressive decline in attentional function with aging in these mice. A very interesting pattern of effects was seen for size of these neurons in the NBM/SI. For the 2N mice, there was a significant reduction in ChAT- immunoreactive cell size as a function of aging, but not for the trisomic mice. The young trisomics and young 2N mice did not differ in neuron size, but for the older animals, the size of NBM/SI BFCNs was significantly larger for the old trisomics than for their 2N counterparts. Although it is not clear why NBM/SI ChAT- immunoreactive neurons do not atrophy with age in trisomics as they do in 2N mice (Fig 5C), one possibility is that this may be due to some sort of compensatory mechanism to increase cholinergic activity to target regions.

These effects of the trisomy on NBM/SI neurons are consistent with two previous studies by our group. In one study, trisomic and 2N mice were examined at 6 months of age, with no genotype effect seen for number, density or size of ChAT- immunolabeled cells in the NBM/SI (Kelley et al., 2014a, b). A second study from our group assessed attention and morphology of NBM/SI neurons in aged Ts65Dn and control mice. Similar to the pattern of effects in the present study, this study reported increased count and size of BFCNs in the NBM/SI in the (aged) trisomic mice (Powers et al., in preparation). The present findings provide pivotal new information to aid in the interpretation of these findings in the older trisomics: They reveal that the genotype effect seen in the old animals in this prior study (Powers et al., 2011) for both BFCN

count and size in the NBM/SI (with increased size and count in the trisomics) is not due to an age-related *increase* in the trisomics, but rather due to an age-related *decline* in the 2N mice, but not the trisomics.

The correlational analyses revealed some interesting relationships between the behavioral and neural measures, providing support for functional links. A borderline significant positive association was seen between ChAT-immunoreactive cell number in the NBM/SI and performance in attention task 2 for the 2N mice (old and young combined); i.e., as cell number increased, performance increased (Fig. 6B). This finding supports the importance of NBM/SI neurons for attentional function and suggests that the aging related decline in attention in the 2N mice may reflect the corresponding loss of NBM/SI neurons.

Our analyses also revealed that for the older animals (genotypes combined), increased BFCN cell size was associated with poorer performance in attention task 2 (Fig. 6A), consistent with one other report from our lab (Powers et al., in preparation). It is not clear whether this correlation merely reflects the fact that the older trisomics both perform more poorly and have larger cells than their 2N counterparts; i.e., that the correlation is an epiphenomenon, or alternatively whether these larger cells are functioning less well, resulting in poor attention. Future studies are needed to make this determination.

Conclusions

The impairment in focused or sustained attention observed in young Ts65Dn mice parallels the attentional dysfunction reported for young individuals with DS (Brown et al., 2003; Cornish et al., 2007; Clark and Wilson, 2003; Wilding and Cornish, 2004), providing further support for the validity of the Ts65Dn mouse as a model for DS. The findings in the aged trisomics are con-

sistent with previous reports that Ts65Dn mice exhibit attentional dysfunction relative to aged 2N mice (Driscoll et al., 2004; Moon et al., 2010) and are consistent with findings in DS humans after the onset of AD-like neuropathology (Della Sala et al., 1992; Foster, 2001; Krinsky-McHale et al., 2008; Levinoff et al., 2004; Pignatti et al., 2005). One main goal of this study was to determine whether attentional dysfunction becomes more pronounced with age in Ts65Dn mice or manifests differently. This study revealed that in tasks requiring focused attention but with a predictable cue onset time, older trisomics were more impaired than young trisomic mice, but that in tasks requiring attention over a variable pre-cue delay, the two age-groups were impaired to a similar degree.

This study also provided the first examination of aging effects on morphology of NBM/SI BFCNs of the Ts65Dn mouse model of DS, and assessed their relationship with the attentional deficits in these animals. It has been hypothesized that the attentional dysfunction seen in Ts65Dn mice may be due to a reduced number of cholinergic neurons in the NBM/SI neurons. However, the present study did not reveal a loss of neurons in the NBM/SI in the trisomic mice (relative to 2N) at either age. For both age-groups, however, the density of these neurons was lower in the trisomic mice than the 2N, which may contribute to the attentional dysfunction seen in both groups. However, the fact that this decreased density did not become more pronounced with aging suggests that the progressive decline in attention seen in the older trisomics must be caused by some other neurological change associated with AD onset. It is also notable that the trisomic mice did not exhibit the normal age-related decline in number and volume of these neurons, as seen in the 2N mice. It's possible that the absence of this age-related atrophy reflects a compensation for dysfunction in the cholinergic system or some other system. Future studies are needed to investigate this. But it is a contribution of this paper to demonstrate

that the attentional dysfunction seen in either young or old trisomics is not due simply to a reduction of cholinergic neurons in the NBM/SI, as many have hypothesized based on early findings in humans with DS (Casanova et al., 1985).

Lastly, our studies shed light on the age-related cognitive deficits seen in normal rodents. Previous studies have shown that with age, attentional function does decline which may be due to decreased cholinergic function (Jones et al. 1995; McGaughy and Sarter 1995; Muir et al.1999). The present study corroborated aging-related attentional dysfunction in normal 2N mice and detected important correlations between attentional performance in the 2N mice and morphology of cholinergic neurons in the NBM/SI. Specifically, we found that both number and size of these neurons decline with aging in the 2N and furthermore that neuron count in the NBM/SI correlated significantly with the attentional ability of these mice. This correlation supports the notion that the loss of these neurons with aging in the 2N mice may underlie their attentional dysfunction.

APPENDIX

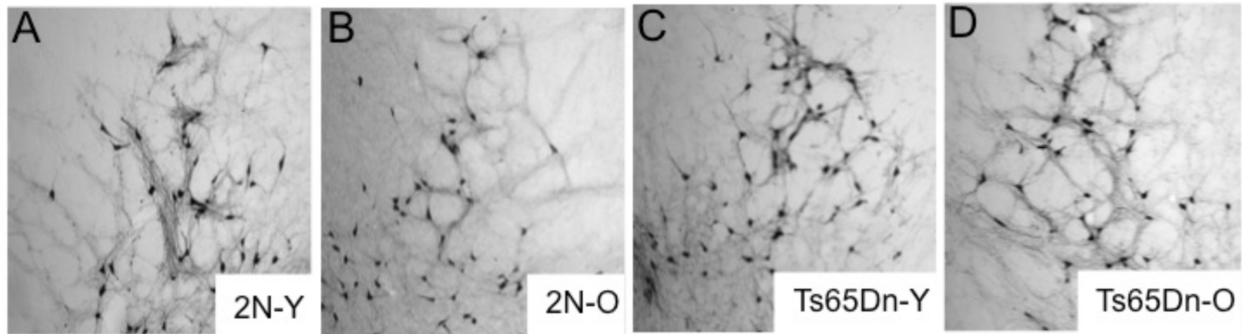

Figure A.1. Photomicrographs of ChAT cells in the NBM/SI: Photomicrographs show the distribution of ChAT- **(A – D)** immunoreactivity within the NBM/SI for the four groups of mice.

GENERAL DISCUSION

The studies presented here have provided further evidence that MCS produces lasting benefits to Ts65Dn mice. Furthermore, we have found that cholinergic neurons within the NBM/SI do not atrophy similarly to that seen in the MS (Cooper et al., 2001; Granholm et al., 2002; Holtzman et al., 1996). Collectively, these findings further contribute to the understanding of changes within the basal forebrain with the onset of AD that arise in DS and furthermore provide support that additional MCS may lessen cognitive function in the case of DS offspring.

Contributions and implications

The study presented in Chapter 3 revealed that Ts65Dn mice exhibit attentional dysfunction as early as 3 months of age, which becomes more pronounced with aging as evident by an increased percentage of omission errors and inaccurate responses in Ts65Dn mice tested from 12-14 months of age, relative to those tested from 3-5 months of age. These findings further validate the Ts65Dn mouse as a suitable model of DS as attentional dysfunction has been revealed in children and adolescents with DS (Brown et al., 2003; Clark and Wilson, 2003; Cornish et al., 2007; Tomporowski et al., 1990; Wilding et al., 2012). A further contribution of this study was to reveal that the Ts65Dn mouse model of DS does not exhibit loss or atrophy of cholinergic neurons within the NBM/SI with the onset of AD-like neuropathology, contrary to BFCNs in the MS. Past research has been interpreted to suggest that the basal forebrain cholinergic system in its entirety atrophies in these mice starting at 6 months of age; however this inference has been reached by solely examining BFCNs within the MS (Cooper et al., 2001; Granholm et al., 2002; Holtzman et al., 1996). This study is the first to examine morphology of cholinergic neurons within the NBM/SI of Ts65Dn mice as a function of age. This finding is

consistent with other reports from our laboratory concerning young (Kelley et al., 2014a, b) and old (Powers et al., in preparation) Ts65Dn mice. Ts65Dn mice did show a reduced density of NBM/SI cholinergic neurons that could contribute to the attentional dysfunction seen at both ages, but the decline in attention across the lifespan in these mice appears to be due to some other neuropathological change. Future work should consider examining other neuropathologies that may contribute to the impairments in DS with age.

Our subsequent studies supplementing the maternal diet with additional choline during pregnancy and lactation revealed substantial improvements of spatial cognition in Ts65Dn offspring that were tested long after this early developmental nutritional manipulation (mean = 15.4 months). These are the first reports of improved spatial cognition with MCS in the Ts65Dn mouse model of DS and further add to the pool of research findings that show benefits of MCS in this mouse model (Moon et al., 2010) as well as normal rodents (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; Wong-Goodrich et al., 2008; Zeisel, 2000) and models of other neural insults (Blusztajn, 1998; Guo-Ross et al., 2002; Moon et al., 2010; Powers et al., 2011; Meck and Williams, 1997b; Meck and Williams, 2003; Yang et al., 2000; Thomas et al., 2000). Furthermore, our studies demonstrate that MCS increased adult hippocampal neurogenesis of the trisomic offspring and offered protection to BFCNs in the MS, both of which were correlated with improved spatial cognition. These results provide exciting new evidence that supplementing the maternal diet with additional choline may represent a safe and effective treatment approach for expectant mothers carrying a DS fetus, as well as a possible means of BFCN neuroprotection during aging for the population at large.

Limitations in our studies

Although the Ts65Dn mouse does mimic pathologies seen in the human disorder, it does not have all the features of DS patients. For example, Ts65Dn mice do not develop Aβ plaques or neurofibrillary tangles like adult humans with DS (Davisson et al., 2005). Aβ plaques and neurofibrillary tangles are neurotoxic, further compromising cognitive functions (Mufson et al., 2003; Isacson et al., 2002; Sendera et al., 2000; Whitehouse et al., 1982). The lack of expression of these pathologies in Ts65Dn mice may reflect the fact that these mice are not trisomic for all of the genes on HSA21, and are trisomic for some genes that are not trisomic in humans with DS (reviewed in Davisson, 2005). Nonetheless, the Ts65Dn mouse model is the most robust and genetically sound to study behavioral and mental deficits associated with DS (reviewed in Davisson, 2005). It has contributed to the further understanding of deficits that arise in the disease as well as treatments that may helpful or harmful for humans with DS. One example includes the test of the drug Piracetam, which was being considered for use in patients with DS after pre-clinical trials; however, after being tested in the Ts65Dn mouse, was removed as a potential therapy because it caused further impairments in learning and memory (Moran et al., 2002).

Future Directions

There is still much to be learned about the degeneration of BFCNs in this mouse model and the relation to cognitive functions. In particular, a few questions arose from the data in chapter 3 that may require further elucidation. One question was why was there a decrease in cell size and number of cholinergic neurons within the NBM/SI for 2N animals that was not observed in the trisomics and furthermore, what could account for the more pronounced attentional dysfunction seen with aging in the trisomic mice. One possibility is that other structures that are

important in attentional functions, such as the frontal cortex (reviewed in Meck and Williams, 2003; McCann et al., 2006), may show damage with age that may lead to further impairments. Another possibility is that may be changes within the NBM/SI that were not detectable with the markers that we utilized. For example, it may be possible that delivery of acetylcholine from the NBM/SI or the bindings of receptors at the target sites are faulty. Future work should examine other possible neuropathologies that may lead to attentional dysfunction and use different measures to detect changes within the NBM/SI.

A few questions remain from our MCS studies. Since we did show that MCS corrected deficiencies in adult hippocampal neurogenesis, the question remains whether we may have corrected ontogenetic neurogenesis and reduced hypocellularity reported at birth in the Ts65Dn mice (Bianchi et al., 2010a; Guidi et al., 2014). Researchers have speculated that adult neurogenesis is an extension of ontogenetic neurogenesis that relies on similar molecular machinery (reviewed in Kuhn and Blomgren, 2011). If this is true, the hypothesis that developmental neurogenesis is corrected with MCS should hold true. Another future direction, based on previous findings from MCS studies in our laboratory (Moon et al., 2010), is to identify what underlies the improved performance in attentional function seen in MCS T65Dn and 2N mice.

Converging evidence continues to suggest that additional CH during gestation and lactation produces lasting improvements in cognitive and affective functioning in normal offspring (Lamoureux et al., 2008; Meck and Williams, 1997a) as well as provides a powerful intervention to help reduce cognitive dysfunction and neurodegeneration in DS and AD (Ash et al., 2011; Moon et al., 2010; Powers et al., 2011; Velazquez et al., 2013). However, prior to recommending that pregnant women increase their choline intake, it is necessary to further elucidate the mechanism(s) underlying these lasting beneficial effects. One future direction, which is currently in its

beginning phases in our laboratory, is to identify the epigenetic changes that underlie the improved cognitive functioning. A start point for this future direction is to look at whether there are differential alterations of DNA methylation in animals whose mothers received additional choline during pregnancy and lactation. DNA methylation is an epigenetic mechanism that alters the expression of genes and is mediated in part by increased maternal CH intake (review in Jiang et al., 2014). Animals that previously participated in behavioral testing provided tissue that will be utilized in this future study to detect change in DNA methylation and gene expression, providing the optimal circumstances to establish functional relationships. This will allow for a determination of what genes are being affected by early CH supplementation and whether these genes are (1) the reason why we see increases in neurogenesis and protection of BFCNS; and (2) whether they are behind the increased performance in spatial memory and (3) whether they are behind the increased performance in attentional function (see in Moon et al., 2010). This study will also fill in the gap of what potential genes are being altered with CH and perhaps aid in determining whether such genes can be targets for other therapeutics during adulthood.

The translation of this work to humans is another future direction. As mentioned in the introduction, one study found benefits in sensory gating in offspring of CH supplemented mothers (Ross et al., 2013). As a starting point, future studies should impose a longitudinal approach and test a variety of cognitive abilities in the adult offspring of CH supplemented mothers. The preliminary stages of additional CH in pregnant mothers has begun in the laboratory of Dr. Barbara J. Strupp and Dr. Marie Caudill at Cornell University. In addition, MCS studies should assess whether DS offspring of pregnant mothers benefit from this intervention in childhood and throughout life. In conclusion, CH recommendations should be reconsidered in order to meet the high demands for optimal fetal development and thus produce favorable health outcomes for

offspring with DS and for the general population.

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