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Abnormal APP, cholinergic and cognitive function in Ts65Dn Down's model mice

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Abstract

We evaluated Ts65Dn Down's syndrome mice and their littermates (LM) at 12, 4, and 12 months of age to determine amyloid precursor protein (APP)-related cellular and biochemical changes associated with cognitive deficits. Ts65Dn mice showed cognitive deficits in the Morris water maze compared to LM mice at 4 and 12 months of age. Ts65Dn, but not LM mice, developed a septohippocampal cholinergic neuronal degeneration of choline acetyltransferase (ChAT)-positive neurons at 12 months of age. These cellular changes were compensated by increases in ChAT enzyme activity of remaining cholinergic terminals in the hippocampus. By 12 months of age, Ts65Dn mice had elevations of APP protein levels in the hippocampus compared to their LM. At this age, both Ts65Dn mice and their LM abnormally responded to cholinergic muscarinic M1 agonist treatment in terms of hippocampal APP, nerve growth factor (NGF), and brain-derived neurotrophic factor (BDNF) levels compared to young adult C57BL/6 mice. In summary, the Ts65Dn mice show developmental and progressive age-related behavioral deficits, hippocampal APP, and cholinergic pathology. The relatively better cognitive spatial performance in LM compared to Ts65Dn mice suggests that high APP levels combined with progressive degeneration of the cholinergic system are critical to the pathology and cognitive deficits seen in Ts65Dn mice.

Keywords: Cognition; Cholinergic transmission; APP processing; NGF expression; Muscarinic agonist; Septohippocampal function

Introduction

The regulation of synaptic amyloid precursor protein (APP) may be a critical factor in both normal brain function and the pathology seen in Alzheimer's disease (AD) and Down's syndrome (DS) (Isacson et al., 2002). It is therefore important to examine animal models with abnormal gene expression or mutated forms of APP with a focus on the dynamic synaptic control of APP, neurotransmitters, and hippocampal functions involved in cognition (Isacson et al., 2002; Kamenetz et al., 2003).

DS; human trisomy 21 is a genetic developmental disorder associated with mental retardation and neuropathological abnormalities; including reduction in neuronal density, dendritic spine development, and synaptic function (Becker et al., 1991; Epstein, 1986; Kaufmann and Moser, 2000; Wisniewski and Rabe, 1986). Cholinergic degeneration also occurs in early adulthood for DS individuals, which is analogous to the degeneration seen in the patients of AD (Casanova et al., 1985; Holtzman et al., 1992; Yates et al., 1983). Ts65Dn mice have segmental trisomy for the distal end of mouse chromosome 16, which shares a large region of genetic homology with the critical region of DS on human chromosome 21 (Davisson et al., 1993). Ts65Dn

Abbreviations: α-APPs, secreted form of amyloid precursor protein; Aβ, amyloid β; AD, Alzheimer's disease; APP, amyloid precursor protein; BDNF, brain-derived neurotrophic factor; CSF, cerebrospinal fluid; ELISA, enzyme-linked immunosorbent assay; LTP, long-term potentiation; NGF, nerve growth factor; TrkA, tyrosine receptor kinase A; ChAT, choline acetyltransferase; p75NGFr, p75 low-affinity NGF receptor.

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mice have been used as an animal model of DS with consistent phenotypic abnormalities, such as developmental delay, hyperactivity, early onset obesity, and reduced learning ability (Davisson et al., 1993; Holtzman et al., 1996; Reeves et al., 1995).

There are several potential neurological and structural substrates that could contribute to the pathogenic process eventually producing the cognitive deficits seen in AD and DS (Isacson et al., 2002). First, many experimental studies demonstrate that memory impairments can be caused by cholinergic neuronal and terminal degeneration (Browne et al., 2001; Lin et al., 1999). Postmortem studies of AD patients show reduced choline acetyltransferase (ChAT) immunoreactivity in the cortex and hippocampus with concomitant reduction in cell size and number of cholinergic neurons in the basal forebrain (Baskin et al., 2000). APP expression and amyloid β [Aβ; aggregate levels increase, whereas secreted APP (APPs)] decrease in the hippocampus and basal forebrain of individuals with AD and DS (Van Gool et al., 1995; West et al., 1995). Platelet APP isoform ratios correlate with reduced cognition in AD, as indicated by Mini-Mental State Examination (MMSE) score (Baskin et al., 2000). Transgenic mice overexpressing the wild type C-terminal fragment of APP also have cognitive disturbance, suggesting that abnormal regulation of APP could be directly involved in the pathology and synaptic dysfunction in both sporadic and hereditary AD (Berger-Sweeney et al., 1999). Experimental loss of cholinergic input to the basal forebrain [including fimbria–fornix lesions and 192-nerve growth factor (NGF)r IgG–saporin lesions] produces abnormal accumulation of APP in the hippocampus (Lin et al., 1998, 1999), indicating a direct relationship between afferent synaptic cholinergic system and APP regulation (Isacson et al., 2002). Interestingly, Sheng et al. (2002) also demonstrated that disruption of afferent corticocortical connections reduces amyloid burden in synaptic terminal fields of the hippocampal dentate gyrus in mutant APP transgenic mice, again suggesting an afferent synaptic control of APP levels.

Changes in trophic factor regulation may contribute to the progressive brain dysfunction seen in AD and DS. NGF and tyrosine receptor kinase A (TrkA) levels are decreased in basal forebrain but increased or unchanged in the hippocampus of individuals with AD (Fahnestock et al., 1996; Goedert et al., 1986; Mufson et al., 1996, 1997; Salehi et al., 1998). These altered levels either imply that retrograde transport of NGF, NGF binding to trkA receptors, or its molecular signals (Fahnestock et al., 1996; Goedert et al., 1986; Mufson et al., 1995, 1997) are reduced. These changes would result in inadequate trophic support of the cholinergic system possibly accelerating its degeneration. Two recent specific studies of transgenic mice progressively expressing NGF antibody support such a concept by an age-related appearance of plaques, tangles, and impaired cognition (Capsoni et al., 2000, 2002a). These deficits were partially ameliorated by early NGF or cholinergic agonist treatment (Capsoni et al., 2002b). We recently reported that cholinergic stimulation by a cholinergic M1 agonist physiologically decreases APP and NGF levels in the hippocampus of normal young adult mice (Seo et al., 2002). In the present study, we investigated altered age-dependent cholinergic synaptic control of APP levels, trophic regulation, and hippocampal function of Ts65Dn mice.

Materials and methods

Experimental groups, M1 agonist treatment, and behavioral testing

Female Ts65Dn and LM mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Ts65Dn mice were generated by repeated crossings of Ts65Dn females to C57BL/6Jei × C3H/HeSnJ (B6EiCSn) F1 hybrid males. All mice were chromosomally genotyped and preexamined by individual opthalmoscopy to check retinal degeneration. Only mice without signs of visual impairment were used in this study.

To determine the progressive change with age in a DS model, we examined Ts65Dn and LM mice (1, 4, and 12 months) for cognitive behavior, cholinergic activity, APP, and trophic factors. This span of ages would by comparison to humans (Becker et al., 1991; Kaufmann and Moser, 2000; Wisniewski and Rabe, 1986; Wisniewski et al., 1985) likely represent infant to middle age since these mice live to an average of 18 months, at which time the pathology and functional deficit are severe. Ts65Dn and LM mice were treated with the muscarinic M1-selective agonist, RS86, to find whether cholinergic stimulation alters the cognitive deficits and the dynamic regulation of the expression of total APP and trophic factors in the hippocampus (Lin et al., 1999; Seo et al., 2002). After drug treatment and Morris water maze test, mice were sacrificed and the brains were removed and dissected on ice. For biochemical analyses, hippocampal tissue was taken from each mouse and used for Western blot and ELISA assays. Separate groups of Ts65Dn and LM mice were also perfused at 12 months of age (n = 6 each group) for histochemical analysis.

RS86 (a gift from Novartis, Switzerland) is a selective muscarinic M1 receptor agonist (Palacios et al., 1986; Rupniak et al., 1992; Wanibuchi et al., 1990). Based on our previous studies (LeBlanc et al., 1999; Seo et al., 2002), animals in all experiments reported here were injected twice daily with the following doses: 1.5 mg/kg im at approximately 9:00 a.m. and 2.0 mg/kg im 8 h later, for eight consecutive days [(1–2 months (n = 5), 4 months (n = 5), and 12 months (n = 20)]. For the control experiment, the same numbers from each group received an equal volume of saline (im) at the same time intervals as the M1 agonist-treated groups.

The behavioral testing was carried out for all mice in the Morris water maze (poly-Track Video Tracking System; San Diego Instruments, San Diego, CA), starting on the third day of drug treatment. A 4-ft tank was filled with water at 18°C and a clear Plexiglas platform was submerged half an inch underwater in the northeast quadrant of the pool. Each mouse received six trials per day for five consecutive days, and each trial lasted a maximum of 60 s. Mice were placed randomly in the tank from one of four fixed points (designated North, South, East, and West) and allowed to swim for 60 s or until they escaped by finding the platform. On the last day of testing, the mice received an additional trial (spatial probe trial), where the platform was removed from the tank and the mice allowed to swim for 60 s. Total swim time (escape latency), total swim distance, swim speed to the platform, and time spent in the target quadrant were recorded for each mouse for each trial.

APP protein detection

The antibody 22C11 (Boehringer Mannheim, Indianapolis, IN) raised against an N-terminal epitope of APP was used to determine the total APP in protein extracts obtained from the brain tissues. The tissue was homogenized using a hand-held homogenizer in cell lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 10 μg/ml Aprotinin, 25 μg/ml Leupeptin, 10 μg/ml Pepstatin, 1 mM PMSF; all protease inhibitors purchased from Sigma, St. Louis, MO) and then sonicated. Homogenates were centrifuged at 14,000 × *g* for 30 min at 4°C. The supernatant was collected and aliquots were stored at −70°C. The protein levels were determined using Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). Samples containing equal amounts of total protein were boiled with SDS sample buffer and electrophoresed on 10% SDS-polyacrylamide gels. Proteins were electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad). Membranes were blocked with 2.5% nonfat dried milk in 0.05 M Tris-buffered saline (pH 7.4) with 0.1% Tween 20 (TBS-T) and then incubated with the 22C11 antibody (1:500) in 1% nonfat dried milk in TBS-T overnight at 4°C. After the incubation with the secondary horseradish peroxidase (HRP)-linked anti-mouse IgG antibody (dilution 1:6000, Jackson Laboratory) in 0.25% nonfat dried milk in TBS-T, the membranes were visualized by enhanced chemiluminescence (Amersham, Arlington Heights, IL) using Kodak X-omat films (Kodak, Rochester, NY). Films of the Western blots were scanned (Scanner UMAX ASTRA 1200S) using Adobe Photoshop (version 5.5, Adobe Systems) and the optical density (OD) of the APP bands was measured using NIH Image (Version 1.61). The relative APP values were calculated by subtracting the background OD-value from the measured OD of the APP bands. The results were confirmed by duplicate measurements of the same sample.

Histological and stereological procedures

Animals were terminally anesthetized by an ip injection of pentobarbital (100 mg/kg), then perfused intracardially with heparin saline (0.1% heparin in 0.9% saline) followed by paraformaldehyde [4% in phosphate-buffered saline (PBS); 100 ml/mouse]. The brains were removed and postfixed for 8 h in the same 4% paraformaldehyde solution. Following postfixation, the brains were equilibrated in sucrose (20% in PBS), sectioned (40 μm) on a freezing microtome, and collected in PBS.

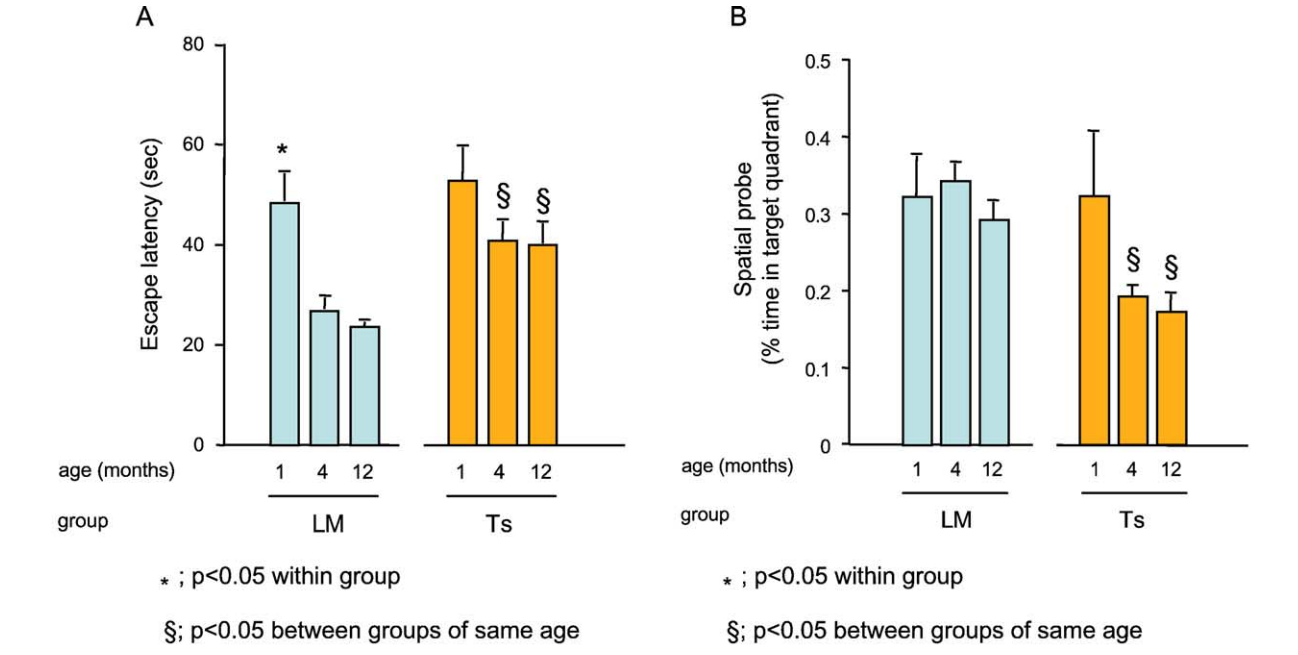
For stereology of immunoreactive cells in free-floating sections, immunoperoxidase and immunofluorescence techniques were employed for visualization of a predetermined fraction of cells. Sections for immunoperoxidase staining were treated for 30 min in 3% hydrogen peroxide, washed three times in PBS, and incubated in 10% Normal Goat Serum (NGS, Vector Laboratories, Burlingame, CA) and 0.1% Triton X-100 in PBS for 60 min prior to overnight incubation at 4°C with the primary antibody (rabbit anti-ChAT, Chemicon, Temecula, CA; 1:750) in 2% NGS and 0.1% Triton X-100. After a 3 × 10-min rinse in PBS, the sections were incubated in biotinylated secondary antibody (goat anti-rabbit, Vector Laboratories; 1:300) diluted in 2% NGS in PBS at room temperature for 1 h. The sections were rinsed three times in PBS and incubated in streptavidin–biotin complex (Vectastain ABC Kit) in PBS for 1 h at room temperature. Following thorough rinsing with PBS, staining was visualized by incubation in 3,3′-diaminobenzidine solution and intensified with nickel (Vector Laboratories). On selected sections, the omission of the primary antibody provided verification of specific staining. After immunostaining, floating tissue sections were mounted on superfrost plus glass slides (Fisher Scientific, Pittsburgh, PA), dehydrated, cleared, and coverslipped.

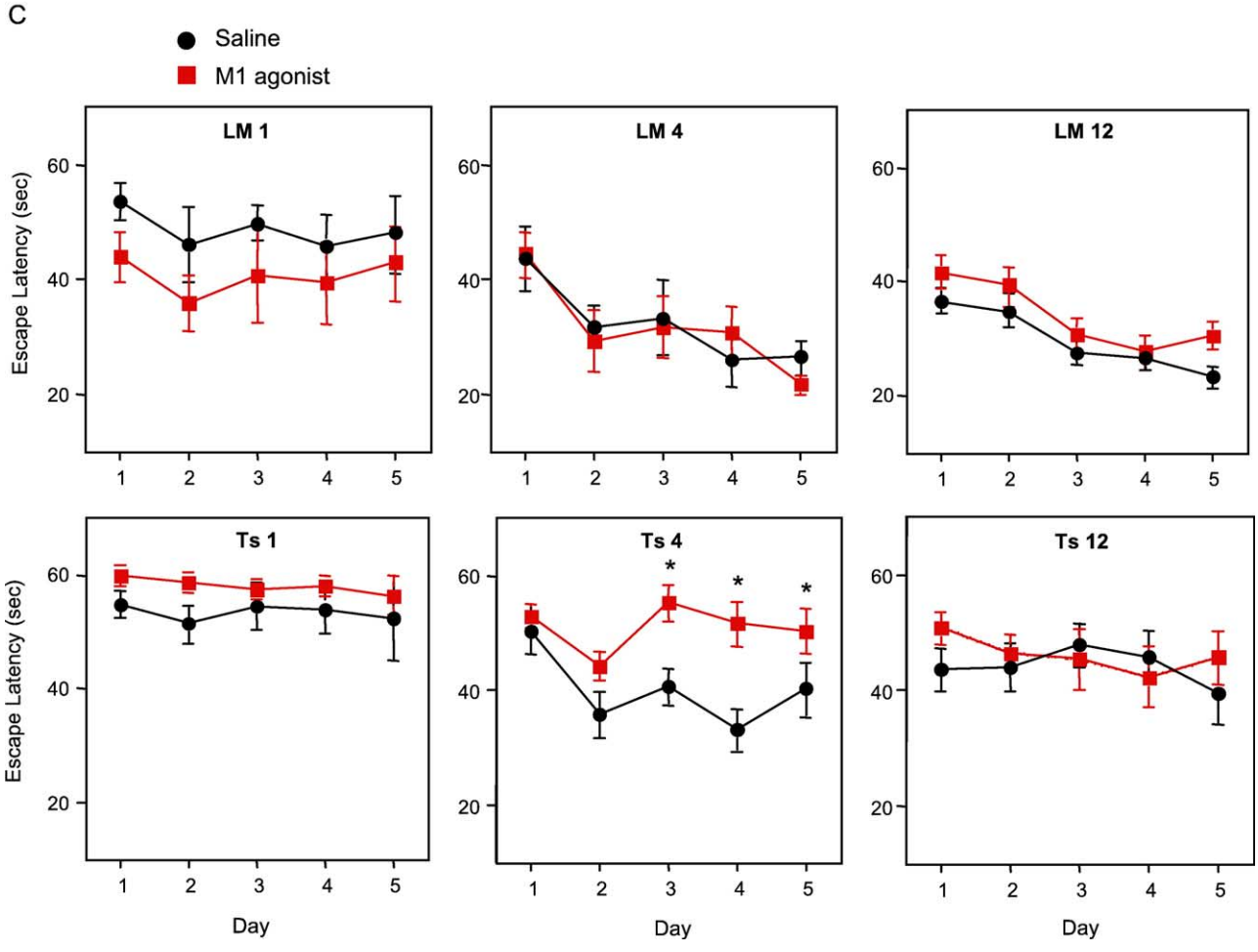
Quantitative stereology was performed on the specimens using an integrated Zeiss (Axioskop 2, Thornwood, NY) microscope, and MicroBrightField (StereoInvestigator, Williston, VT) image capture equipment and software. All ChAT-positive cell bodies were counted in the medial septal nucleus and the medial septum using the above system. ChAT-positive cell soma volumes were measured using a planar rotator probe and the geometric mean was used to interpret the resultant data (Jensen and Gundersen, 1993).

Determination of ChAT activity

ChAT activities were determined as previously described by Fonnum (1975). Briefly, the samples or the standards (Sigma) were incubated at 25°C for 15 min in an incubation mixture (0.5 M sodium phosphate buffer, pH 7.4, 1 M NaCl, 0.2 M EDTA, 80 mM choline chloride, 1 mM eserine, and 10,000 cpm of ^{3}H acetyl CoA/100 μl). One ml stopping solution (0.01 M sodium phosphate buffer, pH 7.4) was then

added, followed by 4 ml of nonaqueous scintillation fluid containing 0.1% kalignost. The contents were vigorously mixed to partition the product (acetylcholine) into the organic phase for counting. ChAT activity was expressed in units of nmol acetylcholine formed per mg protein per hour.





Enzyme-linked immunosorbent assay (ELISA) for NGF or BDNF

The levels of NGF and brain-derived neurotrophic factor (BDNF) in hippocampus were determined using NGF EmaxTM Immunoassay System (Promega, Madison, WI; Zettler et al., 1996) by manufacturer's protocol. Briefly, a polystyrene-coated ELISA plate was incubated overnight at 4°C with the polyclonal anti-NGF antibody or the monoclonal anti-BDNF antibody (1:1000 dilution). Nonspecific binding was blocked by incubating the plate with serum albumin for 1 h at room temperature and washed with 0.1% TBS-T once. A standard curve was created from serial dilutions of known concentrations of NGF or BDNF. Samples were dispensed into the remaining wells and the plate was incubated for 6 h for NGF and 2 h for BDNF at room temperature. The plate was then washed with 0.1% TBS-T 5 times, followed by incubation with the monoclonal anti-NGF antibody (1:4000 dilution) overnight or the polyclonal anti-BDNF antibody (1:500 dilution) for 2 h. After rinsing the plate five times, samples were incubated in the HRP-conjugated tertiary anti-rat antibody (1:2000 dilution) solution for 2.5 h, room temperature for NGF. Samples for BDNF were incubated in the HRP-conjugated tertiary anti-IgY antibody (1:200 dilution) for 1 h at room temperature. After rinsing the plate five times, a TMB solution/peroxidase substrate mixture was added to the wells. The reaction was allowed to proceed for 10 min and stopped with 1 N hydrochloric acid. The ELISA plate was then read using a Labsystem Multiscan Plus plate reader set at 450 nm wavelength.

Statistical analysis

All statistical analyses were carried out using JMP (version 3.1.6, SAS Institute). Data from Western blots and ELISAs were compared between different groups at the different ages with an unpaired Student's t test, a repeated measures ANOVA, two-way ANOVA, and differences between groups were considered statistically significant when $P < 0.05$. Regression analyses were performed with a linear fit for two independent variables (JMP, version 3.1.6, SAS Institute).

Results

Ts65Dn mice display cognitive deficits in spatial performance of the Morris water maze

To determine whether Ts65Dn mice have cognitive deficits and to detect the effect of muscarinic agonists on cognitive function in Ts65Dn mice, we performed a Morris water maze test for five consecutive days. While searching for a hidden platform, Ts65Dn (TS) mice at the age of 4 and 12 months had longer escape latencies compared to littermate (LM) control mice at the same age [Fig. 1A; 4 months: $F(1,8) = 5.24$, $P < 0.05$;12 months: $F(1,17) = 9.29$, $P < 0.007$] and spent less time in the target quadrant than LM mice [Fig. 1B; 4 months: $F(1,8) = 14.59$, $P < 0.005$; 12 months: $F(1,17) = 10.40$, $P < 0.005$]. However, Ts65Dn and LM groups at 1 month of age did not have significant differences in escape latencies. Both Ts65Dn and LM mice at 1 month of age showed longer escape latencies compared to 4 and 12 months of age [Fig. 1A; Ts65Dn: $F(2,14) = 3.96$, $P < 0.04$; LM: $F(2,18) = 11.30$, $P < 0.0007$]. Ts65Dn mice did not show significant learning at any age tested (Fig. 1C), while LM mice showed learning curves in the Morris water maze test at 4 and 12 months of age (Fig. 1C; regression analysis, $r^2 = 0.23$, $P = 0.0158$ at 4 months; $r^2 = 0.28$, $P < 0.0001$ at 12 months). M1 agonist treatment did not significantly improve the cognitive performance in the Morris water maze in any group. On the contrary, Ts65Dn mice at 4 months of age had worse performance after M1 agonist treatment as determined by escape latency at days 3–5 [Fig. 1C; $^{*}P < 0.05$ between groups; day 3: $F(1,8) = 8.78$, $P < 0.01$; day 4: $F(1,8) = 9.68$, $P < 0.01$; day 5: $F(1,8) = 9.12$, $P < 0.01$]. In these water maze behavioral assessments, none of the mice tested showed any sign of visual impairment or motor disabilities.

Ts65Dn mice have higher hippocampal APP levels and abnormalities in the cholinergic system

To investigate whether the cognitive deficit in Ts65Dn is related to APP expression and cholinergic function in the septohippocampal neurons, we determined the APP levels in the hippocampus of Ts65Dn and LM mice, the numbers of ChAT-positive cells in medial septum area, and

Fig. 1. Morris water maze test of Ts65Dn (Ts) and littermate (LM) mice. (A) Escape latency (seconds) in two experimental groups of mice at different ages. Ts mice showed significantly worse cognitive performance than LM controls at 4 and 12 months of age. ($^{*}P < 0.05$ within group, $^{\S}P < 0.05$ between groups of same age). At 1–2 months of age, both Ts65Dn and LM mice had significantly longer escape latencies than parental background control mice at the same age (data not shown; $P < 0.05$), but only LM mice improved their performance by 4 and 12 months of age. (B) Time spent in the target quadrant (spatial probe test). At 4 and 12 months of age, Ts65Dn mice spent significantly less time in the target quadrant than LM mice, indicating a spatial memory disability ($^{*}P < 0.05$ within group, $^{\S}P < 0.05$ between groups of same age). (C) Learning curves in the Morris water maze test for 5 days (TS = Ts65Dn mice, LM = littermate normosomic mice, 1 = 1–2 months, 4 = 4 months, 12 = 12 months; $^{*}P < 0.05$ between groups). Ts65Dn mice did not learn in the Morris water maze at any age tested while LM mice learned at 4 and 12 months of age. In all groups, we could not detect a significant improvement in escape latency or learning curves by M1 agonist treatment (the values are presented as means ± SEM). Statistical analysis was done by two-way ANOVA analysis and Tukey–Kramer post hoc analysis when significant F ratios were present). In these water maze behavioral assessments, none of the mice tested showed any sign of visual impairment or motor disabilities.

ChAT enzyme activity in the hippocampus of Ts65Dn mice. Ts65Dn mice showed significantly higher hippocampal APP levels compared to LM mice at 12 months of age [Fig. 2; $F(1,17) = 238.9$, $P < 0.0001$], which may be attributable to a developmental decrease in APP observed in LM mice. However, at the other ages, hippocampal APP levels were not significantly different between groups of same age. Interestingly, in LM mice, hippocampal APP levels declined significantly with age [Fig. 2; $F(2,18) = 40.32$, $P < 0.0001$], indicating that reduction of the absolute levels of APP may contribute to better cognitive performance during learning (Bimonte-Nelson et al., 2003).

At 12 months of age, Ts65Dn mice showed a reduced number of ChAT-positive neurons in septal area compared to LM mice [Fig. 3; $F(1,5) = 5.79$, $P < 0.05$]. ChAT-positive cell body size changes were not apparent ($P > 0.05$, LM = 1275 ± 15, Ts65Dn = 1273 ± 25). In a size distribution analysis ($n = 5$), however, we found that two animals in the Ts65Dn mice had about 20% less average cell volume whereas the others were in the normal or above range (data not shown). Ts65Dn mice at 12 months of age also showed significantly higher ChAT enzyme activity than LM mice [Fig. 4; $F(1,17) = 7.9$, $P < 0.04$]. Hippocampal ChAT activity in Ts65Dn at 12 months of age was also higher than at 1 and 4 months of age in the same group [Fig. 4; $F(2,15) = 4.9$, $P < 0.05$], indicating that the increase in ChAT activity may be a compensation for the loss of cholinergic neurons in the septal area.

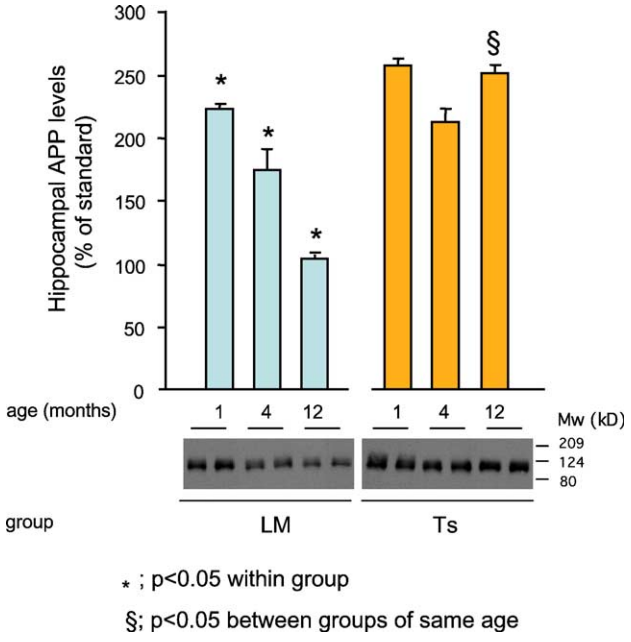


Fig. 2. Hippocampal APP levels in Ts65Dn (Ts) and littermate normosomic control (LM). Ts65Dn mice showed continuously elevated total APP levels in the hippocampus at all ages tested. At 12 months of age, hippocampal APP levels were significantly higher in Ts65Dn mice than LM mice. LM mice showed a decrease of total hippocampal APP levels by age (*$P < 0.05$ within group, $^{§}P < 0.05$ between groups of same age).

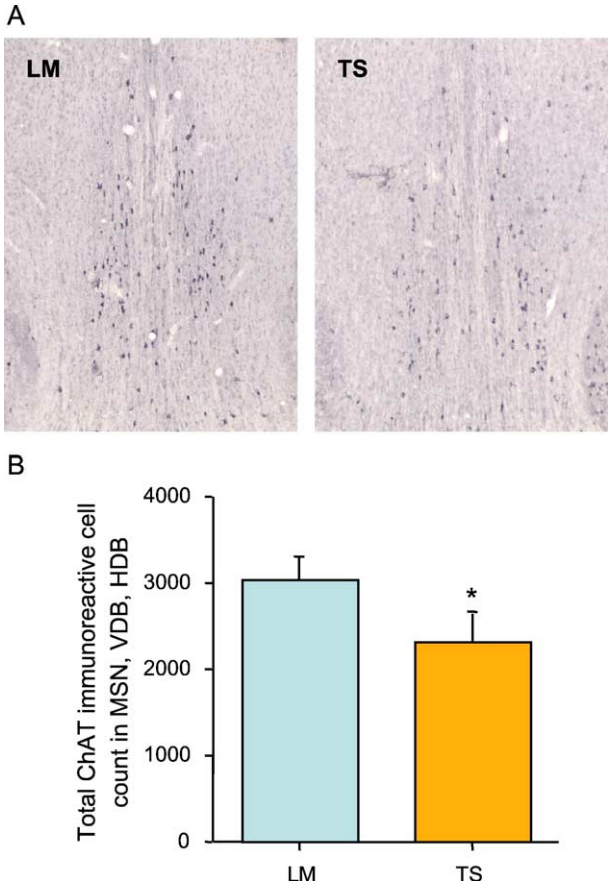


Fig. 3. (A) ChAT-immunoreactive cells in the medial septum of Ts65Dn (Ts) and littermate normosomic (LM) mice at 12 months of age. (B) Ts65Dn mice showed 20% less number of ChAT-positive cells in medial septal nucleus (MSN), vertical limb diagonal band (VDB), and horizontal limb diagonal band (HDB) area compared to LM mice (*$P < 0.05$ between groups).

Ts65Dn and LM mice show age-dependent changes in NGF and BDNF

We determined hippocampal levels of neurotrophic factors such as NGF and BDNF in Ts65Dn and LM mice. Both Ts65Dn and LM mice showed higher hippocampal NGF levels and lower hippocampal BDNF levels than normal inbred mice (parental background control: C57BL/6 × C3H/He) at 1 month of age (data not shown). Hippocampal NGF levels were decreased by age at 4 and 12 months in both Ts65Dn and LM mice [Fig. 5A; Ts65Dn: $F(2,14) = 27.7$, $P < 0.001$; LM: $F(2,18) = 8.78$, $P < 0.002$]. Hippocampal BDNF levels were increased by 4 and 12 months of age in both Ts65Dn and LM mice [Fig. 5B; Ts65Dn: $F(2,14) = 6.7$, $P < 0.05$; LM: $F(2,18) = 5.8$, $P < 0.05$]. Higher NGF protein expression and lower BDNF protein expression at 1 month of age indicate a potential delay of maturation in both Ts65Dn and LM mice. There was an inverse relationship between levels of NGF and BDNF in the hippocampus of both Ts65Dn and LM mice; NGF levels decreased, while BDNF levels increased by age (Figs. 5A and B). No significant differences of NGF and BDNF were detected between Ts65Dn and LM mice at any age.

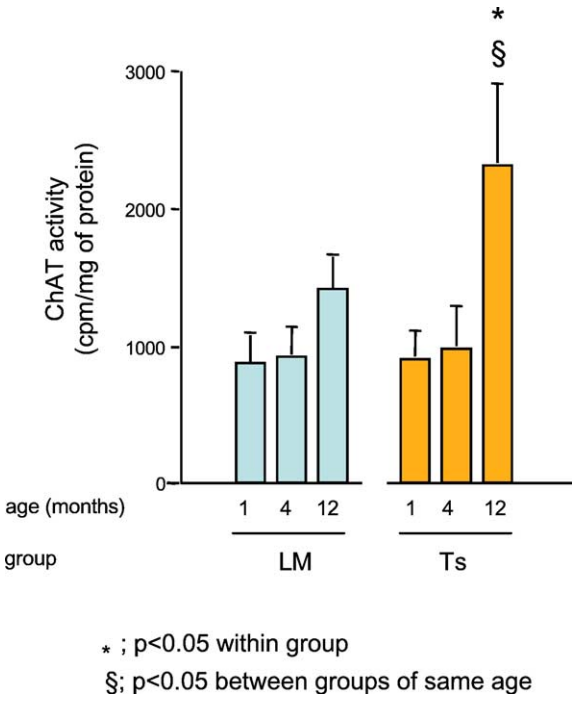


Fig. 4. ChAT activity in the hippocampus of Ts65Dn (Ts) and littermate normosomic (LM) mice are shown as cpm/mg of total protein. Ts65Dn mice showed significantly higher ChAT activity than LM mice at 12 months of age (*$P < 0.05$ within group, $^{§}P < 0.05$ between groups of same age), suggesting compensatory increase of ChAT activity in the remaining terminals in the hippocampus of Ts65Dn mice.

Ts65Dn and LM mice have age-dependent abnormal synaptic responses to an M1-selective cholinergic agonist

Muscarinic cholinergic pharmacological stimulation decreases hippocampal APP levels and NGF levels in normal young C57BL/6 mice (Seo et al., 2002). To examine whether the cholinergic systems of Ts65Dn and LM mice have similar responses to muscarinic agonists, we determined the expression levels of APP, NGF, and BDNF after chronic (7 days) administration of an M1-selective agonist. In contrast to our previous study of normal young adult C57BL/6 mice, M1 agonist increased total APP levels in the hippocampus of both Ts65Dn and LM mice at 12 month of age [Fig. 6A; Ts65Dn: $F(1,14) = 5.48$, $P < 0.03$; LM: $F(1,20) = 5.26$, $P < 0.03$]. We did not detect any of these age-dependent abnormal increases of APP after M1 agonist treatment in parental background inbred mice (C57BL/6 × C3H/He) at 12 and 24 months (data not shown).

M1 agonist treatment did not decrease hippocampal NGF levels (Fig. 6B) or significantly alter hippocampal BDNF levels in either Ts65Dn mice or LM control mice, at 4 and 12 months of age (Fig. 6C), while it decreased hippocampal NGF and BDNF levels in normal young adult BL6 mice (data not shown). These data suggested that both Ts65Dn and LM mice have age-dependent abnormal synaptic responses to M1 agonist at 12 months of age.

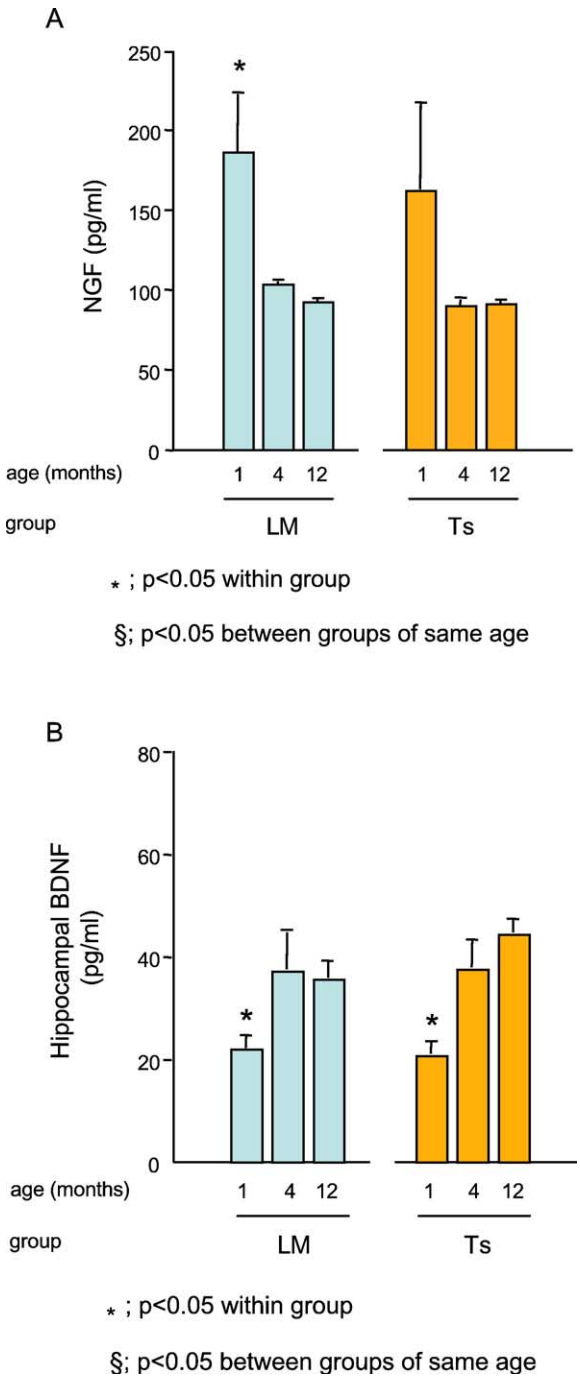
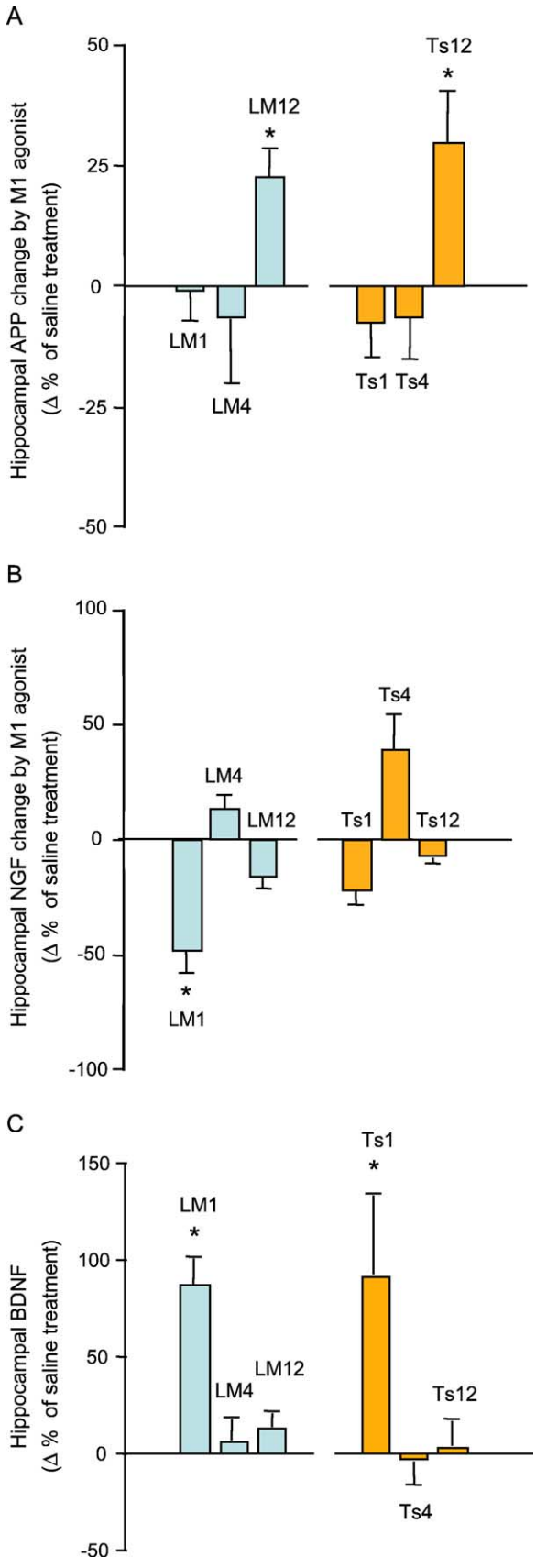


Fig. 5. (A) Hippocampal NGF levels of Ts65Dn (Ts) and their littermate normosomic (LM) mice. Hippocampal NGF levels were reduced at 4 and 12 months in both Ts65Dn and LM mice (*$P < 0.05$ within group). No significant change of NGF was observed between Ts65Dn and LM mice at all ages tested. Hippocampal NGF levels were significantly higher in Ts65Dn and LM mice than in parental strain background control at 1–2 month of age (data not shown, $P < 0.05$). (B) Hippocampal BDNF levels of Ts65Dn and LM mice. Hippocampal BDNF levels tended to increase by age in Ts65Dn and LM mice (*$P < 0.05$ within group). Hippocampal BDNF levels were significantly lower in Ts65Dn and LM mice than in parental strain background controls at 1 month of age (data not shown; $P < 0.05$).



Discussion

In the present study, we determined that (1) Ts65Dn mice at all ages tested (1–2, 4, and 12 months) showed cognitive deficits in Morris water maze test; (2) Ts65Dn mice had an elevated APP expression compared to LM mice at 12 months of age; (3) Ts65Dn mice had cholinergic neuron degeneration and a compensatory increase of ChAT enzyme activity with age; (4) both Ts65Dn and LM mice showed delayed maturation at 1–2 month in terms of spatial memory and trophic factor expression; (5) both Ts65Dn and LM mice at 12 months of age had abnormal synaptic responses to a cholinergic M1 agonist in terms of APP, NGF, and BDNF levels; (6) M1 agonist treatment did not improve cognition in either Ts65Dn or LM mice; and (7) M1 agonist treatment paradoxically increased total hippocampal APP levels. From these and other data, the following multiple factors can be considered as explanations for the cognitive deficits seen in Ts65Dn mice: (a) cholinergic dysfunction including cholinergic neuronal loss and increase of ChAT activity; (b) APP overexpression; (c) aging and development; (d) hippocampal synaptic abnormalities; and (e) overexpression of other factors from trisomic chromosomal segment. These factors and their interactions are considered below in a perspective of accumulating neuropathology with age.

Cognitive impairment and cholinergic dysfunction in Ts65Dn mice

Ts65Dn mice have cognitive impairment in a spatial probe tests (Demas et al., 1996; Reeves et al., 1995), in eight-arm water maze (Hunter et al., 2003) and during context discrimination (Hyde and Crnic, 2001). Ts65Dn mice are born with a normal density of basal forebrain cholinergic neurons, but the density of cholinergic neurons and their cell body size are reduced by 4 months of age (Granholm et al., 2000; Holtzman et al., 1996). This progressive structural decline of cholinergic neurons corre-

Fig. 6. Abnormal synaptic responses in the hippocampus of Ts65Dn (Ts) mice and their littermate (LM) normosomic mice. (A) M1 agonist effects on hippocampal APP levels are shown as percentage of change of APP levels compared with saline treatment group. As previously shown (Seo et al., 2002), young C57BL/6 normal control groups at 1–2 months of age decreased APP levels after M1 agonist treatment. In contrast, hippocampal APP levels were significantly increased by M1 agonist treatment in Ts65Dn and LM mice at 12 months of age. (B) M1 agonist effects on hippocampal NGF levels. One-month-old LM mice showed decreased NGF levels after M1 agonist treatment as shown in normal C57BL/6 mice at 1–2 months of age. In contrast, there was no tendency for a decrease of hippocampal NGF levels in LM and Ts65Dn mice at 4 and 12 months of age. (C) M1 agonist effects on hippocampal BDNF levels. While there was a clear significant increase of hippocampal BDNF levels in normal BL6 mice at 1–2 month of age (data not shown), LM and Ts65Dn mice did not show any alteration of BDNF levels after M1 agonist treatment. Hippocampal APP, NGF, and BDNF levels are shown as percentage of change compared to the saline treatment group (*$P < 0.05$).

lates with a spatial learning deficit (Granholm et al., 2000). A failed retrograde transport of NGF has been reported in Ts65Dn mice by 6 months of age (Cooper et al., 2001). The cell shrinkage and reduced ChAT immunoreactivity in Ts65Dn mice were reversed by NGF infusion directly into the forebrain thereby bypassing the retrograde signaling block (Cooper et al., 2001). In the present study, we found a reduced number of ChAT-immunoreactive cell bodies in the medial septum area in Ts65Dn mice compared to LM mice at 12 months of age (Fig. 3). Interestingly, at that time, ChAT enzyme activity (activity/μg of protein) in hippocampal tissue was increased in Ts65Dn mice (Fig. 4), suggesting a possible compensatory increase of enzyme activity to overcome the loss of cholinergic neuronal input or the loss of cholinergic phenotype. These data show age-dependent progressive cholinergic dysfunction in Ts65Dn mice, which may indeed contribute to the cognitive deficits observed.

Ts65Dn mice and LM mice appear to have a delayed maturation at 1–2 months of age in their acquisition of optimal cognitive performance. Consistent with this interpretation, at 1–2 months both groups had higher hippocampal NGF levels than at 4 and 12 months (Fig. 5A). This developmental retardation may be analogous to human DS (Takashima et al., 1994) and similar to that seen in another DS model, Dyrk1A transgenic mice (Altafaj et al., 2001). The deficits seen in LM mice may for example be a result from a maternal developmental effect or abnormal fostering by a Ts65Dn female (Liu et al., 2000).

Relevance of APP regulation and trophic factors to cognitive impairments

Segmental trisomic Ts65Dn mice, which possess three copies of the APP gene, produce AD- and DS-like pathologies and cognitive impairments that may reflect changes in both APP-related and cholinergic systems (Isacson et al., 2002). The alteration of APP expression, processing, and secretion are closely related to the production of the Aβ peptide (Hardy and Selkoe, 2002), known as a principal component of senile plaques and cerebrovascular deposits in DS and AD (Mattson and Furukawa, 1998; Rossner et al., 1998). It was recently demonstrated that APP processing is closely related to synaptic function (Kamenetz et al., 2003). The formation and secretion of Aβ peptides were modulated by neuronal activity and in turn Aβ depressed synaptic transmission in APP overexpressing neurons (Kamenetz et al., 2003).

AD- and DS-like pathologies can also reflect inadequate responses to trophic factors. In transgenic mice overexpressing NGF antibody, in vivo dysregulation of NGF or its receptor produce AD-like pathologies, including cholinergic degeneration and cognitive declines (Capsoni et al., 2000; Cooper et al., 2001; Dorsey et al., 2002; Lin et al., 1999). Notably, AD patients show reduced TrkA NGF receptor mRNA levels (Mufson et al., 1996), reduced TrkA protein levels (Mufson et al., 1997; Salehi et al., 1998), and typically higher hippocampal NGF protein levels than normal control subjects (Crutcher et al., 1993; Fahnestock et al., 1996). BDNF expression is also altered in AD patient's brain (Fahnestock et al., 2002; Garzon et al., 2002; Holsinger et al., 2000). Abnormal regulation of APP and trophic factors could thus potentially lead to abnormal synaptic neurotransmission, neuronal cell loss, and memory impairments.

Our study covered the ages of 1–12 months of these mice. During this period, we observed relatively high absolute levels of NGF at 1 month in the hippocampus followed by the "middle-age" reduction of absolute NGF levels thereafter. The later stages of cholinergic synapse degeneration lead to paradoxically increased NGF levels in the hippocampus (Cooper et al., 2001; Isacson et al., 2002, and Granholm, personal communication). This appears analogous to disrupted septohippocampal transport of NGF and an abnormal elevation of NGF seen in equivalent late stages of AD (Bartus and Emerich, 1999; Crutcher et al., 1993; Fahnestock et al., 1996; Goedert et al., 1986; Hock et al., 1998, Isacson et al., 2002; Massaro et al., 1994; Mufson et al., 1995; Scott et al., 1995). The parallel normal increases of BDNF levels in the hippocampus during 112 months of age likely reflect the synaptic maturation of other hippocampal pathways, in particular the entorhinal cortex perforant path input and activation of dentate gyrus and interconnected hippocampal subfields (Asztely et al., 2000; Scharfman et al., 2002). It is apparent that NGF and BDNF may influence several synaptic and plastic events that maintain normal electrophysiological responses associated with memory function, including long-term potentiation (LTP) (Isacson et al., 2002). In this perspective, the results reported here indicate how the balance of synaptic trophic regulatory responses are altered in Ts65Dn mice. Consistent with these findings, Bimonte-Nelson et al. (2003) demonstrated a drop in BDNF levels in frontal cortex of the Ts65Dn mice by 6 months of age. The significant loss of cholinergic neurons in the septum in 12 months old Ts65Dn mice (compared to their littermates) was accompanied by a compensatory elevation of hippocampal ChAT activity. This presumably normalizing effect on neurotransmission, occurred at "middle-age" of the mice and in a period when NGF and BDNF concentrations were not yet pathologically altered in the Ts65Dn or LM mice (Bartus and Emerich, 1999; Crutcher et al., 1993; Fahnestock et al., 1996; Goedert et al., 1986; Hock et al., 1998; Isacson et al., 2002; Massaro et al., 1994; Mufson et al., 1995; Scott et al., 1995). Notably, at 12 months of age, both experimental groups had reversed their responses to the cholinergic pharmacological agent in terms of APP levels, while normal mice typically have reduced APP levels after muscarinic M1 (and partly M3) agonist stimulation (Lin et al., 1999; Seo et al., 2002) or learning (Bimonte et al., 2002). At 12 months of age, the mice had elevated APP levels, presumably increasing the risk for amyloid pathology and cognitive damage (Bimonte

et al., 2002; Cooper et al., 2001; Isacson et al., 2002; Lin et al., 1999; Seo et al., 2002).

Interestingly, the regulations of APP and NGF by M1 muscarinic agonist treatment are closely correlated in normal C57BL/6 mice (Seo et al., 2002), indicating a link between APP and trophic factors in the normal dynamic synaptic function of the corticohippocampal system (Isacson et al., 2002). APP levels depend in part on cholinergic function, age-related factors, and cognitive decline (Bimonte et al., 2002). Loss of cholinergic control can lead to increased APP levels, which are associated with cognitive decline (Lin et al., 1999). Moreover, the process of learning in a swim maze is associated with reductions of hippocampal APP levels (Bimonte et al., 2002). Hippocampal APP levels also correlate with escape latencies in water maze testing of rats with cholinergic lesions (Lin et al., 1999). Consistent with specific elevation of hippocampal APP levels, Ts65Dn mice also had longer escape latencies at 12 months of age compared to LM mice. Conversely, total hippocampal APP levels in LM mice progressively decreased and escape latencies improved. These data suggest that elevated total hippocampal APP levels in Ts65Dn mice may reflect synaptic dysfunction of APP regulation associated with cognitive deficits.

Hippocampal cholinergic system responses to cholinergic stimulation in Ts65Dn mice

Cholinergic muscarinic M1 agonists reduce total hippocampal APP levels and increase levels of secreted APP in 192 IgG–saporin lesioned rat and normal BL6 mice (Lin et al., 1999; Seo et al., 2002). M1 agonist stimulation also decreases hippocampal NGF levels in normal young BL6 mice (Seo et al., 2002). The alterations in NGF levels produced by M1 agonist treatment and affecting APP changes in hippocampus suggest a dynamic regulation of trophic factors by cholinergic input (Seo et al., 2002). Cholinergic muscarinic stimulation also increases levels of BDNF expression in the hippocampus (French et al., 1999; Poulsen et al., 2002). In the present study, the hippocampal APP levels in Ts65Dn mice increased after M1 cholinergic agonist treatment in Ts65Dn and LM mice at 12 months of age. Presumed abnormal synaptic responses to cholinergic M1 agonist stimulation were also detected in terms of hippocampal NGF and BDNF levels in Ts65Dn mice and their LM mice at 4 and 12 months of age. In summary, both LM and Ts65Dn mice have abnormal hippocampal responses to muscarinic agonists by 12 months of age.

Hippocampus-related abnormalities have been reported in Ts65Dn mice. Basal production of cAMP in the hippocampus of Ts65Dn mice is impaired (Dierssen et al., 1996). Cooper et al. (2001) showed higher p75 low-affinity NGF receptor (p75NGFr)-immunoreactive fiber density at 6 months and a lower p75NGFr-immunoreactive fiber density reading at 18 months in the molecular layer adjacent to the inferior blade of the dentate granule cell layer (Cooper et al., 2001). There are also changes of morphology of hippocampal dendrites in Ts65Dn mice by 12 months (Granholm et al., 2000; Seo et al., unpublished data). These data suggest that Ts65Dn mice have both pre- and postsynaptic dendritic abnormalities of p75-immunoreactive cells in hippocampus. Ultrastructural studies have also demonstrated significantly fewer asymmetric synapses and larger synaptic apposition lengths in the temporal cortex of Ts65Dn mice (Kurt et al., 2000) and a reduced number of synapses in the hippocampus of Ts65Dn mice (Seo et al., unpublished data). Ts65Dn mice have a reduced LTP and increased LTD compared to age-matched controls (Siarey et al., 1997). Deficiencies in synaptic transmission of the central β-noradrenergic system were also reported in Ts65Dn mice (Dierssen et al., 1997). Taken together, abnormal structure and cellular and synaptic regulation of hippocampus may explain deficits in cognitive performance of Ts65Dn mice.

However, it is still possible that the other factors on the trisomic chromosomal segment are also related to hippocampal abnormalities and cognitive deficits in Ts65Dn mice. For example, the MMU16 area in the segment of trisomy of mice chromosome 16 includes genes such as SOD, Olig, and Dyrk (Davisson et al., 1993). The overexpression of any of these gene products may also contribute to developmental and neuropathological abnormalities in the hippocampus of Ts65Dn mice.

In conclusion, dynamic regulation of multiple factors such as high APP levels, cholinergic cell loss, and synaptic abnormalities along with aging may produce poor performance in Morris water maze for the segmental trisomic (Ts65Dn) mice. Additionally, this study suggests that a cholinergic pharmacological or related treatment must be evaluated on the basis of the age and/or stage of disease, or as a responsiveness based on cholinergic synaptic function.

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