

Estrogen Alters Amyloid Precursor Protein As Well As Dendritic and Cholinergic Markers in a Mouse Model of Down Syndrome

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ABSTRACT: Individuals with Down syndrome (DS) develop the pathological hallmarks of Alzheimer's disease at an early age, later followed by memory decline and dementia. Women with DS are twice as likely to undergo early menopause, and levels of estradiol correlate with onset of cognitive decline in these women. We have demonstrated that a mouse model of DS, mice with segmental trisomy of chromosome 16 (Ts65Dn), develop a significant deficit in both reference and working memory as young adults (6–10 months of age), coupled with phenotypic loss of cholinergic neurons in the basal forebrain and altered growth factor levels. In the present study we examined cholinergic and dendritic markers in the hippocampal formation and levels of the amyloid precursor protein (APP) in different brain regions of Ts65Dn mice treated with estradiol for 60 days. The density of the dendritic marker Map2 was significantly decreased in the hippocampal formation of middle-aged trisomic mice (9–15 months old), and the density of cholinergic neurites (acetylcholinesterase [AChE] histochemistry) was also decreased in specific layers of the hippocampus. Treatment with 17 β -estradiol alleviated the decreases in Map2 and AChE staining, but had no effect on full-length APP levels in the hippocampus. In contrast, a main effect of treatment on APP levels in the striatum was noted, with significant elevations observed in controls and trisomics. These findings demonstrate that estrogen can alleviate deficits in cholinergic and dendritic elements in the hippocampal

formation and further strengthens the rationale to explore estrogen replacement therapy in women with DS. © 2003 Wiley-Liss, Inc.

KEY WORDS: amyloid precursor protein; amyloid β -peptide; acetylcholine; hippocampus; cortex; neurodegeneration; Alzheimer's disease; Down syndrome; dementia; microtubule-associated proteins

INTRODUCTION

As the life span of individuals in the western hemisphere increases, an exponential increase in age-related diseases such as Alzheimer's disease (AD) has been reported. Several different mutations, particularly in the gene for the amyloid precursor protein (APP) and its associated processing enzymes, presenilins, have been connected to subtypes of familial AD (for review, see, e.g., Price, 1999; Selkoe, 1999). Interestingly, the gene for APP is located on chromosome 21 in humans, and individuals with Down syndrome (DS), who have a partial or complete trisomy of chromosome 21, have been found to develop most pathological markers for AD early in life (Yates et al., 1980; Casanova et al., 1985; Wisniewski et al., 1985; Mann and Esiri, 1989; Sendra et al., 2000; for review, see Head et al., 2001). Because AD families exhibit a higher rate of DS cases and vice

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versa, it has been postulated that the two diseases are related (see, e.g., Petronis, 1999). The Ts65Dn mouse that is trisomic for a segment of the murine chromosome 16 has been used as a model for DS as well as recently also for early onset AD (Davisson et al., 1990; Crnic and Pennington, 2000a; Granholm et al., 2000; Cooper et al., 2001; Bimonte et al., 2002). This animal model mimics the genetic profile and phenotype of DS, and can be used to further the understanding of the pathogenic process of AD and DS as well as facilitate development of novel therapeutics for these disorders.

One of the hallmarks for AD as well as DS is the loss of cholinergic neurons in the basal forebrain (BF; see Whitehouse, 1998, Sendera et al., 2000), a group of neurons known to be important for learning and memory both in humans and in animal models (Perry et al., 1978; Kasa et al., 1997; Cummings et al., 1998; Lawrence and Sahakian, 1998; Whitehouse, 1998; see also Isacson et al., 2002). Ts65Dn (trisomic) mice are born with normal amounts of BF cholinergic neurons (Holtzman et al., 1995, 1996) and normal spatial memory (Crnic and Pennington, 2000). However, when Ts65Dn mice are 6–8 months of age, they are subjected to a progressive degeneration of cholinergic phenotype in BF neurons, similar to that seen in AD and DS patients, coupled with a decline in spatial working and reference memory (Holtzman et al., 1995, 1996; Demas et al., 1996, 1998; Granholm et al., 2000; Hyde et al., 2001; Cooper et al., 2001; Bimonte et al., 2002; Hunter et al., 2003). A loss of cholinergic neurites in the hippocampal formation around the same age has also been reported (Cooper et al., 2001).

Amyloid precursor protein (APP) is a large transmembrane pre-protein that is present in most neurons of the CNS (reviewed in Selkoe, 1999). APP has been shown to be located predominantly in the cell membrane of neurons (Haass et al. 1992; see also Neve et al., 2000); it has therefore been suggested that APP is involved in either cell-to-cell adhesion or signaling, or both (Perez et al., 1997; Mattson et al., 1999; Neve et al., 2000). One prominent theory in AD research is that it is the processing of APP into the aggregating amyloids (of which the most toxic form is called A- β 1–42) that initiates the cascade leading to plaques, neuronal death, and memory loss (Selkoe, 1999; Price, 1999; Neve et al., 2000). Other investigators claim that it is the loss of the neurotrophic soluble amyloid precursor protein (sAPP) fragments during abnormal APP processing that leads to memory loss and neuronal death, as sAPP has been shown to be necessary for memory initiation and other cognitive processes (see, e.g., Mileusnic et al., 2000), and APP levels have been shown to be correlated with cognitive performance in rats (Lin et al., 1999). A third prominent theory of AD says that amyloid is not the primary system that starts the disease process, but instead alterations in the internal structure of the microtubules of neurons, including the formation of paired helical fragments (PHFs), leads to early disruption of axonal transport and, eventually, cell death (see, e.g., Jellinger et al., 1991; Muketova-Ladinska et al., 2000). Even though they do not develop actual amyloid plaques, Ts65Dn mice also show increased levels of full-length APP mRNA throughout the brain (Reeves et al., 1995), because APP is one of the genes located on the trisomic gene segment, both in humans with DS and in Ts65Dn mice (Reeves et al., 1995).

Hence, we will be able to test the neurobiological mechanism of the neurotoxic effects amyloid, without the complex interaction from actual plaque formation.

Microtubule-associated proteins (MAPs), such as Map2 and tau, are important for the integrity of the axonal transport system in both the peripheral and central nervous systems (see, e.g., Buee et al., 2000; Garcia and Cleveland, 2001), and one of the pathological processes that occur in AD patients is a displacement of these proteins in favor of abnormal PHFs (Ashford et al., 1998). Recent data suggest that one of the “normal” functions of APP may be as a membrane cargo receptor for kinesin-1 (the microtubule “motor”), and the APP protein may thus be an integral protein for axonal transport (see Kamal et al., 2000). Deletion of the APP gene in mice has been shown to lead to disruption of Map2 staining in the hippocampus, suggesting an important connection between these two proteins during normal neuronal signaling in this brain region (Seabrook et al., 1999). Thus, immunostaining with microtubule-associated proteins would demonstrate alterations in axonal transport and neuronal “infrastructure”, and perhaps also an important connection between APP and microtubule-associated proteins as neurodegenerative disease evolves. Such studies have not been performed on Ts65Dn animals to our knowledge.

Cognitive impairment during AD has been shown to be diminished by estrogen replacement therapy (ERT), but results have varied depending on the type of memory tests conducted and the choice of patients in the study (Henderson et al., 1994; Schneider et al., 1995; Birge, 1996; Tang et al., 1996; Gandy and Duff, 2000). Some investigators do not report beneficial effects of estrogen in any memory tasks in women with AD (Mulnard et al., 2000), leaving this important question unresolved to date. Estradiol treatment has not been tested in elderly women with DS, even though they are twice as likely to undergo early menopause than non-DS women (Carr and Hollins, 1995; Schupf et al., 1997; Cosgrave et al., 1999) and show a decline in cognitive function linked to menopause (Patel et al., 2001). Estradiol modifies the function and morphology of hippocampal neurons *in situ* and *in tissue culture*. In particular, it has been shown that estradiol treatment during development increases the volume of the CA1 and CA3 hippocampal regions, as well as improves spatial navigation behavior (Isgor and Sengelaub, 1998). Dramatic effects of estrogen have also been observed on density of hippocampal synapses (Woolley and McEwen, 1992), dentate gyrus neurogenesis (Tanapat et al., 1999), the strength of hippocampal long-term potentiation (LTP; Cordoba Montoya and Carrer, 1997), and spatial tasks thought to require an intact hippocampus (Daniel et al., 1997; Packard and Teather, 1997; Luine et al., 1998; Bimonte and Denenberg, 1999; Fader et al., 1999). However, studies have not been conducted, to our knowledge, on effects of estrogen on hippocampal morphology in an animal model of DS.

We recently demonstrated that ERT enhances cognitive performance as well as cholinergic markers and growth factor levels in middle-aged trisomic mice (Granholm et al., 2002). In the present study, we wanted to expand previous findings and investigate whether ERT during middle-age or aging can alleviate observed

alterations in hippocampal measures in trisomic animals, as well as affect the levels of APP in different brain regions.

MATERIALS AND METHODS

Animals

Trisomic mice contain an extra chromosome (Chr) with a segment of the mouse Chr16 region homologous to human Chr 21 attached to the centromeric region of mouse Chr 17. The trisomy is maintained by mating female carriers of the partial trisomy (males are sterile) to C57Bl/6 jeicher \times C3H/HeSnJ F1 males (Davisson et al., 1990). For the present studies, female mice were bred in the laboratory of Dr. Linda Crnic from stock obtained from the Jackson Laboratories. The mice were genotyped by fluorescence in situ hybridization (FISH), using a probe for the telomeric end of mouse Chr 16 (Korenberg et al., 1999). Animals with retinal degeneration (rd) due to homozygosity for the mutation carried by C3H mice (detected by amplifying the rd mutation (Granholtm et al., 2000) were discarded. Mice were maintained on a 12/12 light/dark cycle (light onset 7:00 A.M.), had free access to tap water and NIH 5K67 autoclaved mouse chow, and were group housed until sacrifice.

Estrogen Treatment

Fourteen gonadally intact female trisomic mice and 11 normosomic littermates (9–15 months of age; mean age 11.3 months) received a subcutaneous 60-day-release pellet containing 0.25 mg 17 β -estradiol (Innovative Research of America, Sarasota, FL). Twelve trisomic and 12 normosomic animals received a Sham operation identical to the surgery for the estrogen pellet insertion. For pellet insertion, animals were anesthetized with Avertin (500 mg/kg i.p.), an incision was made in the dorsal neck region, one pellet was placed subcutaneously in each mouse, and then the skin was sutured. The study was run in two duplicates. For ease of discussion, hereafter trisomic mice that received sham and estrogen pellet surgery are designated trisomic SHAM (Tris-Sham) and trisomic EST (Tris-EST), respectively, while normosomic littermate controls receiving Sham and estrogen pellet surgery are designated normosomic SHAM (Norm-Sham) and normosomic EST (Norm-EST). In another set of experiments, 12 trisomic animals (9–15 months old) received an EST pellet, and 12 served as Sham-operated controls, while 6 normosomics received an EST pellet and 6 received a Sham surgery. This second set of animals were treated identically but were perfused with 4% paraformaldehyde in phosphate-buffered saline (PBS) at the time of sacrifice for morphological studies of the hippocampal formation (see below).

Western Blots for APP Protein Detection

The antibody 22C11 (Boehringer-Mannheim, Indianapolis, IN) raised against the N-terminal epitope of APP was used to determine the APP level in protein extracts obtained from brain

tissue (Lin et al., 1998, 1999). 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 μ g/ml aprotinin, 25 μ g/ml leupeptin, 10 μ g/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride (PMSF); all protease inhibitors purchased from Sigma Chemical Company, St. Louis, MO) and then sonicated until all viscosity was lost. Homogenates were centrifuged at 14,000g for 30 min at 4°C. The supernatant was collected and aliquots were stored at -70°C . Samples containing equal amounts of total protein were boiled with sodium dodecyl sulfate (SDS) sample buffer and electrophoresed on 10% SDS-polyacrylamide gels. Proteins were electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA). 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 22C11 antibody (1:500) in 1% nonfat dried milk overnight at 4°C. After the incubation with the secondary horseradish peroxidase (HRP)-linked anti-mouse IgG antibody (dilution 1:6,000, Jackson Laboratories, Bar Harbor, ME) in 0.25% nonfat dried milk, the membranes were visualized by enhanced chemiluminescence (ECL) (Amersham, Arlington Heights, IL), using Kodak X-omat films. The hippocampus from a young rat was used as the same internal standard for all blots, and values were thus expressed as a percentage of the standard in order to compare values between different batches of animals.

Densitometric Analysis

Quantification of APP immunoreactive bands was performed using densitometry. Films of 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. Representative samples of APP blots are shown in Figure 1.

Statistical Analysis

All statistical analyses of Western blot data were carried out using Statistica (version 6, Statsoft, Tulsa, OK). Data from Western blot and enzyme-linked immunosorbent assay (ELISA) were analyzed using analysis of variance (ANOVA) with Tukey-Kramer post hoc analyses (Statview), and differences between groups were considered statistically significant when $P < 0.05$.

Immunohistochemistry for Dendritic Markers

At the end of the experiment, animals were deeply anesthetized with chloral hydrate (600 mg/kg, i.p.) and perfused through the aorta with 0.9% saline followed by 4% paraformaldehyde. The brains were rapidly removed from the skull, postfixed with 4% paraformaldehyde, and transferred the next day to 30% sucrose in PBS. Using a microtome (D-6900 Heidelberg HM 400), 40- μ m sections were obtained from the hippocampal formation of each

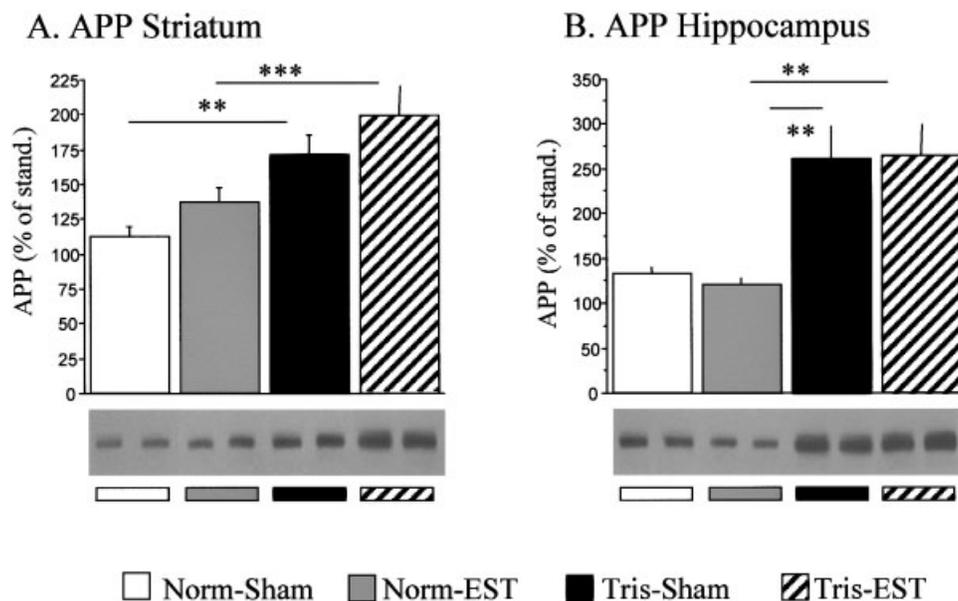


FIGURE 1. Effects of estrogen on amyloid precursor protein (APP) levels. Both estrogen treatment and trisomy increased APP levels in the striatum (A), but in the hippocampus only genotype affected APP levels (B). For striatal levels, a 2-between (Genotype \times Treatment) ANOVA showed that there was a marginal effect of treatment ($P < 0.06$) and a significant effect of genotype ($P < 0.001$). Post

hoc analysis confirmed these differences; when collapsed over genotype, estrogen did indeed elevate APP levels ($*P < 0.04$; A). Sample gels beneath each graph demonstrate representative differences among the four groups in striatal and hippocampal levels of APP, respectively.

animal Sections were processed for free-floating immunohistochemistry, using antibodies against Map2 (Sigma). Immunohistochemistry was performed according to our standard protocol (Backman et al., 1996). Controls included sections where the primary antibody was omitted, as well as a pre-incubation of the primary antibody with the appropriate antigen. The sections were washed in Tris-buffered saline (TBS), incubated with the secondary antibody reacted with the ABC solution (Vectastain; Vector Laboratories) and diaminobenzidine (Sigma) (DAB, 0.0300 g/dl imidazole-acetate buffer solution; Fisher Scientific). Sections were mounted on glass slides and coverslipped. All sections were viewed using a Nikon Eclipse 600 light microscope.

Acetylcholinesterase Histochemistry

Sections were washed 3 times in PBS and were then incubated with 5 mM NaOAc pH 5.0, 1 mM glycine, 0.2 mM CuSO_4 , 1.15 mg/ml acetylthiocholine iodide (Sigma) overnight. The sections were washed 3 times in PBS, air dried, and mounted with Permount. They were studied in a Nikon Eclipse light microscope and subjected to densitometry as described below.

Image Analysis of Staining Density

Immunofluorescence staining intensity of the sections was determined by use of NIH Image[®] software (written by Wayne Rasband). Image[®] can be used to measure area, average gray value, as well as path lengths and angles of cellular components. Spatial calibration is supported to provide real world area and length measurements. A density

calibration was performed against an optical density calibration curve that takes into account and subtracts the background from each section that is measured. The gray scale value is within the range of 0–256, where 0 represents white and 256 black. Thus, more staining results in higher number on the arbitrary scale. Images were captured with a Cohu video camera (4990 series) coupled to a Nikon Optiphot microscope (20 \times objective) connected to a Macintosh Quadra 840 AV computer. Mean staining intensities of Map2 and AChE labeling were thus obtained and subtracted from background staining. The staining density subtracted from background was evaluated on 6 sections that were separated by $\geq 300 \mu\text{m}$ per brain. The first section of each region was randomly picked; systematic sampling was then employed (every sixth section was used for image analysis of staining density). The densitometry measurements were performed blindly by two independent investigators, and results were combined to reflect the mean value for each brain. (For further information regarding the image analysis system used, see Backman et al., 1996.)

RESULTS

Estradiol Levels in Trisomic and Normosomic Animals

As expected, estrogen treatment increased serum estradiol levels in Normosomic and trisomic mice. The serum estradiol levels in the normosomic sham (17 ± 4.33 , SEM, $n = 6$) group were

comparable to those reported during the estrus phase (8–10 pg/ml), and Normosomic EST levels (70 ± 20.9 , $n = 5$) were close to the range of those found during the proestrus phase (50–60 pg/ml) of the estrous cycle in normal, cycling adult female mice (Grasso and Reichert, 1996; see also Granholm et al., 2002). For the trisomic SHAM mice, the estrogen levels were 7.5 ± 1.45 ($n = 5$) and estrogen raised the levels in the trisomic EST group to within the normal range (90 ± 20 , $n = 6$). The overall effect of estrogen treatment was statistically significant [$F(1,13) = 6.6$, $P < 0.05$], but the effect of genotype was not. The estradiol data were recently published in a separate publication including the behavioral data from these animals (see Granholm et al., 2002).

Effects of Estrogen on Striatal and Hippocampal APP Levels

Relative APP levels in the striatum

A 2-between ANOVA (Genotype \times Treatment) showed a marginal effect of treatment [$F(1,43) = 3.69$, $P < 0.06$] and a significant effect of genotype on relative APP levels [$F(1,43) = 18.929$, $P < 0.001$]. Post hoc analysis showed a significant increase of APP levels in the striatum of EST treated trisomics and normosomics when collapsed over genotype ($P < 0.05$), and a significant elevation of striatal APP levels in trisomics versus normosomics ($P < 0.001$; see Fig. 1A). The estrogen effect was insignificant within each genotype group. Estrogen further appeared to increase differences in APP levels between trisomics and normosomics, so that post hoc analysis showed a greater difference between these two groups in estrogen treated animals ($P < 0.001$; see Fig. 1A). Sample blots for both striatum and hippocampus are shown beneath each bar graph in Figure 1.

Relative APP levels in the hippocampus

A 2-between ANOVA (Genotype \times Treatment) did not show any treatment effects in hippocampal APP levels [$F(1,31) = 0.034$, $P = 8547$] but a significant effect of genotype [$F(1,31) = 23.328$, $P < 0.001$]. Hence, while estrogen appeared to increase APP levels in the striatum (Fig. 1A), such treatment effect was not found in the hippocampus in either of the genotype groups (Fig. 1B).

Dendritic and Cholinergic Markers in the Hippocampus

Our previous studies have demonstrated beneficial effects of estradiol on hippocampal-dependent cognitive tasks in trisomic animals (see, e.g., Granholm et al., 2002). Therefore, we wanted to examine whether estradiol had direct effects on the morphology of hippocampal neurons. We used two different markers for hippocampal integrity: Map2, which is a microtubule-associated protein (Geddes et al., 1994), and AChE histochemistry, to label cholinergic input to the hippocampus. Twelve trisomic Sham animals (Tris-Sham), 12 trisomic estrogen (Tris-EST), 6 normosomic Sham (Norm-Sham), and 6 normosomic estrogen animals (Norm-EST) were used for morphological studies. These animals

were the same age as the animals used for APP analysis above (9–15 months). They were all females. After staining of every sixth section for each marker, we performed a double blind investigation of the staining density within different subregions of the hippocampus for the two markers. The most dramatic alterations in hippocampal morphology were seen with the microtubule antibody (Map2; Fig. 2A–C). Trisomic mice exhibited a significant decrease in Map2 labeling, including less staining within each dendrite, and fewer labeled dendrites. This decrease appeared in all hippocampal layers, and was particularly prominent in CA1 and in the dentate gyrus (Fig. 2). The dentate gyrus, the CA3, and the CA1 were outlined using the NIH Image system (see Materials and Methods), and staining to background ratio was assessed. Densitometry analysis showed a significant decrease in Map2 staining density in all three regions of the hippocampus (Fig. 3A). This decrease in Map2 staining was alleviated with estradiol treatment (Figs. 2C and 3A; mean values for the different groups are shown in the figure legend). Similar to the estrogen-mediated upregulation of the microtubule associated protein, there was also a significant increase in the CA1 region of Tris-EST animals for AChE histochemistry, suggesting that estrogen had an effect on cholinergic terminals in this region in trisomic mice as well (Fig. 3B; $P < 0.05$). Microphotographs of AChE labeling are shown in Figure 2D–F. Densitometry showed that estrogen had significant effects on AChE-labeled neurites only in the CA1, and not in the CA3 or the dentate gyrus (Fig. 3B).

DISCUSSION

Our data in the present communication demonstrated that immunolabeling for microtubule-associated proteins (Map2) and cholinergic neurites (AChE histochemistry) was deficient in the hippocampus of trisomic mice, and that estrogen treatment normalized these two markers of hippocampal integrity. Furthermore, we found that estradiol treatment increased levels of the APP holoprotein in the striatum but not the hippocampus in both groups. APP levels were significantly increased in trisomic, compared with normosomic, mice in both brain regions. Finally, estrogen treatment increased the genotype effect in the striatum.

Even though we did not see any effects of estrogen on APP levels in the hippocampus, it was interesting to note that estrogen increased APP levels in the striatum in the present study. Further studies of the function and structure of the striatum in trisomic mice are warranted, because some aspects of motor function are altered in these mice (see, e.g., Klein et al., 1996; Costa et al., 1999; Hyde et al., 2001a). The elevated APP levels in striatum with estrogen described in the present report are supportive of another study, showing that estrogen increases the mRNA for APP695 in estrogen replacement of ovariectomized aged female rats (Chao et al., 1994). On the contrary, Shi et al. (1998) found that estrogen attenuated overexpression of APP in a focal ischemia model, suggesting that APP mRNA expression is altered depending on the

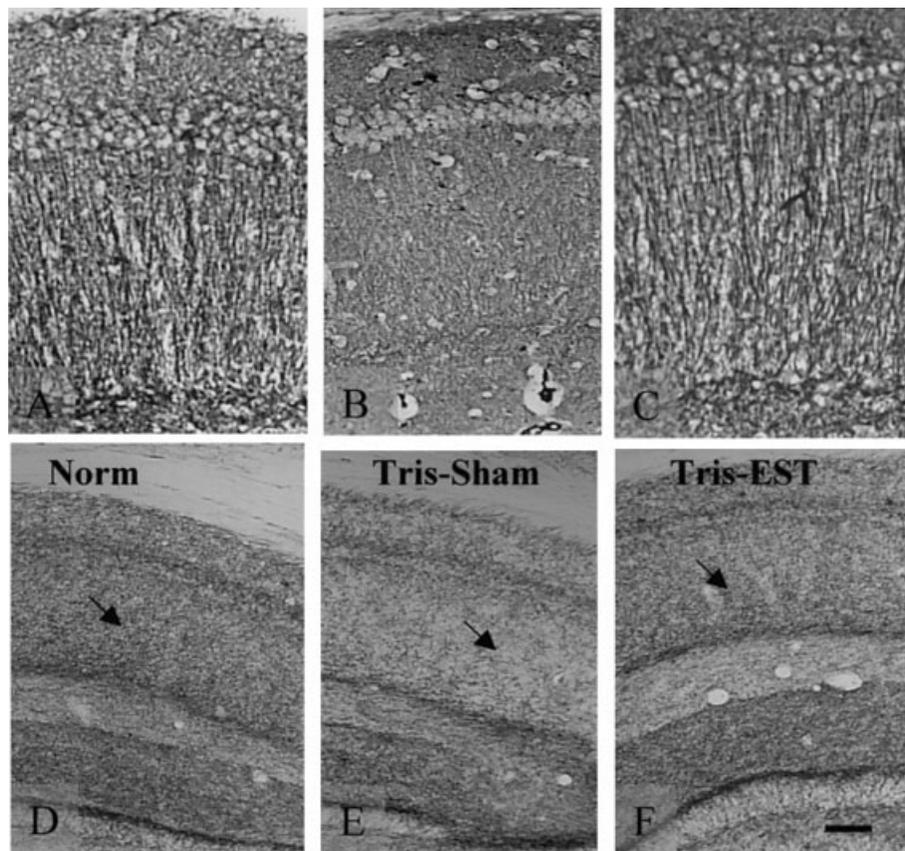


FIGURE 2. A–C: Dendritic and cholinergic labeling is altered in the hippocampus of trisomic animals. Microtubule-associated protein (Map) 2 immunohistochemistry in the CA1 region of the hippocampus (A–C). A: Normosomic animal. B: Trisomic sham animal. C: Trisomic EST animal. Note that there is a significant reduction in Map2 labeling in the trisomic mouse (middle), compared with normosomic animals, but that this decrease appears to be normalized by estrogen treatment. D–F: Histochemistry using acetylcholinesterase

(AChE) staining, showed that trisomic animals had a regional decrease in AChE-labeled neurites in the CA1 region of the hippocampal formation (arrows). The micrographs demonstrate AChE staining in the CA1 from a normosomic animal (D), a trisomic sham animal (E), and a trisomic EST animal (F). There was a subtle reduction in AChE staining, with pockets of staining loss, particularly in the CA1 layers (CA1, see arrows) in the trisomic sham animals, compared with the other two groups. Scale bar in C = 75 μ m.

type of injury endured. It has not previously been demonstrated, to our knowledge, that estrogen also alters APP protein levels in rodents. Our study thus extends previous findings, suggesting that estrogen enhances APP expression both at the mRNA and protein level, regardless of genotype of the animal. The APP protein appears to have a complex interaction with MAPs, since animal models with APP overexpression (e.g., the Ts65Dn mouse described herein), as well as those with an APP deletion (see Seabrook et al., 1999), exhibit structural loss of the microtubular network, coupled with a significant Map2 decrease. The notion that “medium” levels of APP are needed for normal neuronal function in the hippocampal formation carries over to our recent finding in young and aged rats, showing that optimal cognitive performance on a working memory task was linked to “medium” levels of APP in the hippocampus as well (Bimonte et al., 2002b). Several studies have indicated that APP is necessary for normal cognitive function (see Lin et al., 1999; Mileusnic et al., 2000), and it is therefore important to explore levels of APP, rather than just the cleavage process of this protein into different products such as amyloid. Most stud-

ies have, however, indicated that the strongest effects of estrogen on APP are related to processing rather than synthesis of the holoprotein (Jaffe et al., 1994; Chang et al., 1997). Jaffe and collaborators reported a decreased production of the damaging A- β products when estrogen was applied to a cell line with estrogen receptors in tissue culture, suggesting a shift in the processing towards sAPP rather than amyloid peptides. Thus, it will be important in future studies to explore the relationship between estrogen and APP processing in our animal model, particularly in light of reports demonstrating that estrogen may reduce the production of neuronal amyloid peptides (Xu et al., 1998). Individuals with DS develop significant accumulations of extracellular A- β at an early age, suggesting that either overproduction or deficient processing of APP may be related to the memory deficits that occur later on (Leverenz and Raskind, 1998).

A recent study by Cooper and collaborators (Cooper et al., 2001) demonstrated that axonal transport of NGF is disrupted in trisomic mice. Our Map2 findings are consistent with that observation, as Map2 labeling tends to be deficient when the infrastruc-

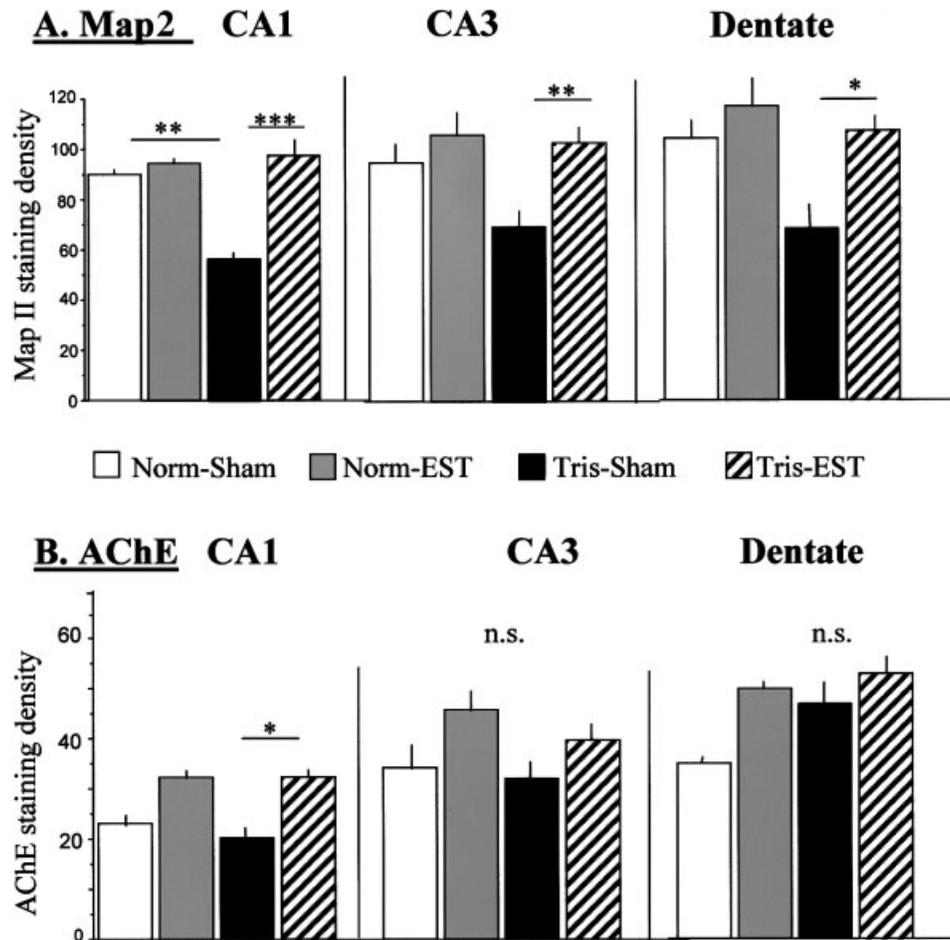


FIGURE 3. Densitometry of microtubule-associated protein (Map) 2 and acetylcholinesterase (AChE) staining in the hippocampus. Image analysis showed significant alterations in trisomic animals in the Map2 immunostaining in all regions of the trisomic hippocampus (A), while cholinergic markers appeared to be significantly affected by the trisomy only in the CA 1 region (B). As can be seen here, estrogen normalized the density of Map2 staining in all three regions (mean values were not statistically different from the normosomic controls), as well as the density of AChE staining in the CA 1 (B). Norm-Sham, normosomic sham-operated animal; Norm-EST, Normosomic estrogen treated; Tris-Sham, trisomic sham-operated; and Tris-EST, trisomic estrogen treated. Mean Map2 staining levels for

the different groups were for the CA1: Norm-Sham, 89 ± 3.7 ($n = 6$); Norm-EST, 94 ± 3.7 ($n = 6$); Tris-Sham, 55.9 ± 5.3 ($n = 12$); and Tris-EST, 97 ± 7 ($n = 12$). For the CA3, the Map2 values were: Norm-Sham, 94.7 ± 7.6 ($n = 6$); Norm-EST, 108 ± 12 ($n = 6$); Tris-Sham, 70 ± 6.6 ($n = 12$); and Tris-EST, 100.7 ± 7.9 ($n = 12$). For the dentate gyrus, the Map2 staining densities were: Norm-Sham, 103.9 ± 23 ($n = 4$); Norm-EST, 110 ± 2.3 ($n = 4$); Tris-Sham, 68 ± 11 ($n = 6$); and Tris-EST, 106 ± 10.3 ($n = 6$). Mean values for AChE staining in the CA1 were: Norm-Sham, 24 ± 0.9 ($n = 4$); Norm-EST, 33 ± 2 ($n = 6$); Tris-Sham, 20 ± 2.3 ($n = 12$); Tris-EST, 32 ± 2.7 ($n = 12$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

ture of the microtubular network is lost, for example after colchicine administration to the hippocampus (Geddes et al., 1994). Other animal models that have shown a significant loss of Map2 immunolabeling include traumatic brain injury (Adlard et al., 2000), and apolipoprotein E-deficient mice (Veinbergs et al., 2000). Both of the latter studies demonstrated that treatment with antioxidants or microtubule-stabilizing compounds (taxol) alleviated Map2 deficiencies, as well as cognitive loss in these two different animal models, indirectly supporting the notion that microtubule associated proteins are crucial for normal hippocampal functioning (such as memory processing), similar to the findings reported in our study herein. We found that estrogen treatment upregulated Map2 immunostaining in the hippocampus of tri-

somic mice (Fig. 3A). This suggests that estrogen may act on dendritic structure, and restore some of the microtubule-associated proteins that were lost in trisomic animals before treatment. The effects of estrogen on Map2 immunolabeling may be due to the proven antioxidant effects of estrogen (see, e.g., Inestrosa et al., 1998), since other antioxidants apparently have similar effects on the hippocampal microtubular network (see above).

In a previous study, Packard and Teather (1997) found that direct infusion of estradiol into the hippocampal formation increased memory in ovariectomized rats and the effect was attributed to direct hippocampal actions by estrogen. Our current findings of MAP2 immunolabeling with estrogen treatment provide a structural substrate for such behavioral changes. Other studies have

also shown that synapse density fluctuates during the estrous cycle in female rats, with the highest densities seen during proestrus (or when estradiol levels are highest; see, e.g., Woolley and McEwen, 1992). Since the cytoskeleton is essential for neuronal signaling and maintenance of function, it is not surprising that microtubule associated proteins are altered in the trisomic mouse, since deficits in hippocampus-dependent learning have been observed in these mice previously (see, e.g., Demas et al., 1996, 1998; Hyde et al., 2001b; Bimonte et al., 2002a).

How does estrogen work? Steroid hormones act through a group of high-affinity receptors that regulate transcription by binding to hormone response elements (HRE) within the promoters of target genes, which are organized with nuclear proteins to form chromatin (Archer et al., 1995; see also Behl et al., 2000). It remains unknown whether the effects on Map2 protein described here are caused by direct estrogen-Map2 interactions or through an indirect regulatory mechanism. It is well known that estrogen acts as an antioxidant, most likely through “nongenomic” effects, directly on hippocampal neurons without going through other systems (McEwen et al., 1997; Inestrosa et al., 1998). It has also been shown that the estrogen response element is located in the promoter region of the NGF gene and in the genes of related members of the neurotrophin family, such as BDNF (Watson and Milbrandt, 1989). There are multiple studies that have shown that estrogen elicits some of its effects on cognition by upregulating the use of endogenous NGF (see, e.g., Gibbs, 1998; Simpkins et al., 1997). One likely hypothesis for the memory loss observed in trisomic mice is thus that an early deficiency in cholinergic neuron function leads to a downregulation of *trk A* high-affinity NGF receptors, in turn leading to altered retrograde transport of this growth factor (see Cooper et al., 2001).

Another plausible explanation for the cholinergic deficits in trisomic mice and individuals with DS is that the overproduction of APP and/or amyloids, or, alternatively hyperphosphorylation of tau for example, affect the microtubular network early in the disease process, and that this in turn affects the transport of growth factors to the cell bodies of cholinergic neurons, since MAP-associated proteins are definitely involved in the disease process. With time, this may lead to degenerative alterations in cholinergic neurons, such as the loss of neurites observed in the present study, and finally cell death. Map2 is one of the proteins that is sensitive to insult in the adult and aged CNS, and early alterations in Map2 integrity have been observed for example in APP deficient mice (Seabrook et al., 1999). It is especially interesting to note that animals with APP deletion react with deficits in microtubular networks, since APP has been proposed as the culprit in both AD and individuals with DS and AD. Again, this suggests that it is the loss of “normal” APP and sAPP function that causes most of the damage in AD and normal aging, and not the accumulation of amyloid plaques. Our animal model, the Ts65Dn mice, indirectly prove that the plaques are not the primary cause for AD-related cell death, since we have found significant loss of cholinergic neurons (Granhholm et al., 2002), as well as age-related memory loss (Hyde et al., 2001b; Bimonte et al., 2002b), without plaque formation in this animal model. APP has been found to be involved indirectly in

microtubular transport, functioning as a “cargo protein” for other proteins (see, e.g., Kamal et al., 2000). The trisomic mouse model appears to be an excellent model for exploring relationships between hormone replacement protocols and amyloid processing, since the mice both have an elevated APP expression, cholinergic cell loss, and deficient endogenous hormones, similar to individuals with DS.

In conclusion, our studies provide a structural basis to explain the functional deficits of trisomic mice observed in working and spatial reference memory paradigms (see, e.g., Demas et al., 1996, 1998; Hyde et al., 2001a,b; Bimonte et al., 2002a). In future studies, it will be interesting to determine which specific function the overexpression of APP has in the striatum of trisomic mice, as well as determining the interaction between the Map2 protein with for example A- β fragments and phosphorylated tau. Collectively, the findings presented here suggest that the trisomic mouse serves as an excellent model for AD and also for Alzheimer-like pathology in DS individuals. Future studies should be focused on processing of the APP protein into smaller damaging or protective peptides as well as correlations with neuroprotective treatment paradigms, in order to clarify the disease process and potential therapeutic avenues for AD and DS patients.

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