Effect of Estradiol and Soy Phytoestrogens on Choline Acetyltransferase and Nerve Growth Factor mRNAs in the Frontal Cortex and Hippocampus of Female Rats (44393)

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Abstract. **We report here the effects of oral micronized estradiol and soy phytoestrogens on uterine weight, choline acetyltransferase (ChAT) and nerve growth factor (NGF) mRNAs in the frontal cortex and hippocampus of ovariectomized young and retired breeder rats. Within each age category, 15 bilaterally ovariectomized rats were randomized equally into three groups: control (OVX), estradiol (E2), and soy phytoestrogens (SBE). The OVX rats were fed a casein/lactalbumin-based control diet; the E2 rats were fed with the control diet with added estradiol; and the SBE rats were fed with the control diet with added soy phytoestrogens. After 8 weeks of treatment, blood, uteri, frontal cortex, and hippocampus were collected at necropsy. Results showed that the uterine weights and serum estradiol concentrations were significantly higher in the E2 group compared with those in the OVX and SBE groups. In the hippocampus of young rats, E2 treatment resulted in significantly higher NGF mRNA levels than no treatment (OVX), and NGF mRNA levels in the SBE group were intermediate between the E2 and OVX groups. ChAT mRNA levels were significantly higher in the frontal cortex of E2 and SBE-treated retired breeder rats compared to OVX retired breeder rats. There were no differences among treatment groups for ChAT mRNA levels in the frontal cortex of young rats and in the hippocampus of both young and retired breeder rats. Our data suggest that soy phytoestrogens may function as estrogen agonists in regulating ChAT and NGF mRNAs in the brain of female rats.** [P.S.E.B.M. 1999, Vol 221]

A growing body of evidence has suggested that cholinergic neurons are essential for learning and memory processes (1, 2). Alzheimer's disease is strongly associated with decreased ChAT activity and loss of cholinergic neurons (3, 4). The exact mechanisms responsible for Alzheimer's disease have not been elucidated, but a number of biomarkers including choline acetyltransferase (ChAT) activity and nerve growth factor (NGF) have been shown to be important for the function of cholinergic neurons (5). Postmenopausal estrogen treatment is associated with reduced risk for Alzheimer's disease and appears to relieve symptoms of Alzheimer's disease and to preserve cognitive function in women (6, 7), which suggests that estrogen deficiency may be at least partially responsible for the neurodegeneration in Alzheimer's disease (8). The mechanisms that lead to neurodegeneration under the condition of estrogen deficiency are still unknown. Current evidence indicates that estrogen deficiency results in decreased ChAT activity, and ChAT and NGF mRNA, which can be reversed by estrogen replacement (2, 5, 9, 10). Most studies concerning ChAT activity and mRNA have been focused on basal forebrain (5). Some evidence indicates that ChAT in the cortex and hippocampus is not equally affected by es-

This work was supported by a grant from Protein Technologies International, Inc. (St. Louis, MO) and Grant P01-HL45666 from the National Heart, Lung, and Blood Institute (Bethesda, MD).

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Received July 27, 1998. [P.S.E.B.M. 1999, Vol 221]
Accepted December 14, 1998.

0037-9727/99/2212-0118$14.00/0

trogen deficiency (2). In addition, presence of cholinergic neurons and ChAT mRNA in these two brain regions has been reported (11–14). These data suggest the possibility that ChAT mRNA levels in the cortex and hippocampus may play a role in determining ChAT activity in these brain regions.

Estrogen replacement appears to have a protective effect on cognition; however, commonly used estrogens may increase the risk of breast and uterine cancers, and have unpleasant side effects for some women (15, 16). It is important to find compounds that retain the beneficial effects of estradiol and conjugated equine estrogens on the brain, cognition, and Alzheimer's disease, but do not have cancer-promoting effects on the breast and uterus, and do not have other adverse effects. Soy phytoestrogens are among the promising candidates for this purpose because they have no significant proliferative effects on the breast and uterus (17), but retain the beneficial estrogenic effects on the cardiovascular system (18). Until now, their effect on brain biomarkers related to cognitive function and Alzheimer's disease have not been reported.

Establishing a small animal model that can mimic menopausal women will facilitate the investigation of the potential beneficial effects of estradiol and soy phytoestrogens on cognition after menopause. Retired breeders (8–10 months old) were selected as the animal model because their reproductive function begins to decline, and we think that ovariectomized retired breeder rats may mimic postmenopausal women better than ovariectomized young adult rats. Aged rats (24 months old) may not be a good model because of the possibility that irreversible neurodegeneration may have occurred to aged rats, and estradiol cannot restore the lost neurons. Our first objective was to test the hypothesis that estrogen replacement may affect local levels of ChAT and NGF mRNA in the frontal cortex and hippocampus in ovariectomized young adult rats and retired breeders. Our second objective was to examine the hypothesis that soy phytoestrogens may act as estrogen agonists in regulating choline acetyltransferase and NGF mRNAs in the frontal cortex and hippocampus in ovariectomized young adult and retired breeder rats.

Materials and Methods

Materials and Reagents. 17 β-estradiol (Estrace™) was purchased from Mead Johnson Laboratory (Princeton, NJ), and a soy phytoestrogen concentrate, containing 66% (on dry basis) isoflavones with 43% genistein, 21% daidzein, and 2% glycitein, was provided by Protein Technologies International (St. Louis, MO). GeneAmp RNA PCR kit was purchased from Applied Biosystems (Foster City, CA). High Pure PCR purification kit was purchased from Boehringer Mannheim (Indianapolis, IN). Select G-50 STE columns were purchased from 5 Prime 3 Prime (Boulder, CO). Trizol Reagent was purchased from Life Technologies Inc. (Grand Island, NY). Hybond-N membrane, Kodak Biomax MS film, and α-^{32}P-dCTP (3000 μCi/mol) were purchased from Amersham Lifescience (Arlington Heights, IL).

Animals. In this experiment, 15 young adult rats (2–3 months old, weighing 180–200 g) and 15 retired breeders (8–10 months old, weighing 300–360 g) were purchased from Harlan Sprague-Dawley, Inc. The rats were housed individually and maintained on a 12:12 hr light/dark cycle with access to food and water *ad libitum.* All procedures done on the animals were approved by the Animal Care and Use Committee of Wake Forest University.

Within each age group, rats were randomized into three groups based on body weight, and both young and retired breeder rats were bilaterally ovariectomized. Beginning 3 days after surgery, animals in the control group (OVX) were fed a casein/lactalbumin-based diet (young rats: 25 g/day and retired breeders: 40 g/day); the estradiol group (E2) was fed the control diet (young rats: 25 g/day and retired breeders: 40 g/day) with estradiol equivalent to a woman's dose of 2 mg/day (2 mg/1800 cals); and the soy phytoestrogen group (SBE) was fed the control diet (young rats: 25 g/day and retired breeders: 40 g/day) with phytoestrogens equivalent to a woman's dose of 150 mg total isoflavones/day (150 mg/1800 cals), respectively (see Table I for diet composition). At the end of the 8 weeks of treatment, the animals were euthanized with pentobarbital (100 mg/kg). Blood samples were collected at necropsy by cardiac puncture, and serum samples were used to determine estradiol and phytoestrogen concentrations. Brains were removed and processed for the analyses of the biomarkers.

Brain Tissue Collection. Brains were removed from the skull immediately after decapitation. Frontal cortex and hippocampus were dissected and frozen in liquid nitrogen. The frozen samples were then stored at −70°C until RNA isolation.

RNA Isolation. Total cellular RNA was isolated from brain tissues with TRIzol reagent (GIBCOL, BRL). Briefly, frozen brain tissues were transferred into 15-ml centrifuge tubes containing cold TRIzol reagent (1 ml Reagent/50 mg tissue) and homogenized immediately using a Tekmar's SDT homogenizer. The homogenized samples were incubated at room temperature for 5 min. Chloroform (0.2 ml/ml TRIzol reagent) was then added to the samples, and the centrifuged tubes were shaken vigorously by hand for 15 sec. After 3 min of incubation at room temperature, the samples were centrifuged at 12,000*g* for 15 min at 4°C. The colorless upper aqueous phase was then collected into a new centrifuge tube and mixed with 0.5 ml isopropyl alcohol per ml TRIzol reagent. After 10 min of incubation at room temperature, the samples were centrifuged at 12,000*g* for 15 min at 4°C. Finally, the resulting pellets were mixed with 1 ml 75% ethanol/ml TRIzol by vortexing and then centrifuged at 7500*g* for 5 min at 4°C. The resulting pellets were washed with 75% ethanol one more time. The RNA pellets were then dried and dissolved in diethyl pyrocarbonate-treated water at 55°C for 10 min. The relative purity of isolated RNA was assessed spectrophoto-

Table I. Diet Composition

Ingredient	Control diet g/100 grams	E_2 diet g/100 grams	SBE diet g/100 grams
Casein	10.00	10.00	10.00
Lactalbumin	10.00	10.00	10.00
Dextrin	30.00	30.00	30.00
Sucrose	26.50	26.50	26.50
Alphacel	10.00	10.00	10.00
Lard	5.00	5.00	5.00
Safflower oil	1.00	1.00	1.00
Complete vitamin mix[a]	2.50	2.50	2.50
Ausman-Hayes mineral mix[b]	5.00	5.00	5.00
Composition:			
Protein (% of calories)	19.5	19.5	19.5
Carbohydrate (% of calories)	64.6	64.6	64.6
Fat (% of calories)	15.9	15.9	15.9
Saturated (% of calories)	6.0	6.0	6.0
Monounsaturated (% of calories)	6.5	6.5	6.5
Polyunsaturated (% of calories)	3.4	3.4	3.4
Cholesterol (mg/Calorie)	0.02	0.02	0.02
Calcium (mg/Calorie)	1.87	1.87	1.87
Phosphorus (mg/Calorie)	1.30	1.30	1.30
Estradiol (mg/Calorie)	—	0.0011	—
Phytoestrogens (mg/Calorie)	—	—	0.0833

[a] The vitamin mix contains vitamin A palmitate (1.8 g/kg mix), DL-α-tocopheryl acetate (2.5 g/kg mix), i-inositol (5.0 g/kg mix), riboflavin (1.0 g/kg mix), menadione sodium bisulfite complex (2.25 g/kg mix), p-aminobenzoic acid (5.0 g/kg mix), niacin (4.5 g/kg mix), pyridoxine HCl (1.0 g/kg mix), thiamin HCl (1.0 g/kg mix), calcium pantothenate (3.0 g/kg mix), vitamin B_{12} (0.1% trit. in mannitol, 1.35 g/kg mix), biotin (0.02 g/kg mix), folic acid (0.09 g/kg mix), ascorbic acid, coated (90 g/kg mix), choline chloride (75 g/kg mix), and vitamin D_3 (0.2 g/kg mix).

[b] The mineral mix contains calcium phosphate (tribasic, 355.269 g/kg mix), potassium carbonate (260.19 g/kg mix), sodium chloride (162.37 g/kg mix), magnesium sulfate (143.89 g/kg mix), potassium phosphate (dibasic, 67.71 g/kg mix), ferrous sulfate (exsiccated, 7.868 g/kg mix), manganese sulfate (1.351 g/kg mix), zinc chloride (0.91 g/kg mix), cupric sulfate (0.29 g/kg mix), potassium iodide (0.077 g/kg mix), chromium acetate (0.048 g/kg mix), sodium fluoride (0.023 g/kg mix), and sodium selenite (0.004 g/kg mix).

metrically, and the ratio of A260 nm to A280 nm exceeded 1.9 for all preparations.

RT-PCR. RT-PCR was employed to detect the presence of NGF and ChAT mRNAs in the brain samples. The primers used for ChAT, NGF, and β-actin were selected from previous publications (19, 20). The nucleotide sequences of 5′- and 3′-primers for ChAT are 5′-GAGAAGACAGCCAATTGGGTC-3′ and 5′-TTCATCCTCGTTGGACGCCAT-3′, respectively. The 5′-primer sequence for NGF is 5′-CCAAGGACGCAGCTTTCTAT-3′, and the 3′-primer sequence for NGF is 5′-CTCCGGTGAGTCCTGTTGAA. The primer sequences for β-actin are 5′-TCATGAAGTGTGACGTTGACATCCGTAAAG-3′ (5′-primer) and 5′-CCTAGAAGCATTTGCGGTGCACGATGGAGG-3′ (3′-primer). The RT-PCR protocol was modified from the procedure reported by Pan *et al.* (21) by optimizing the annealing temperature for these primer pairs (65°C) and shortening the extension cycle time to 40 sec. The modified PCR cycle conditions were 94°C for 1 min, 65°C for 1 min, and 72°C for 40 sec, followed by a final extension at 72°C for 3 min. The specificity of the RT-PCR products was confirmed by sequencing analysis. Briefly, RT-PCR products were purified with the High Pure PCR Product Purification Kit. About 60–125 ng purified PCR products and 4.2 pmoles of sense primers were subjected to fluorescent automated sequencing analysis performed on the Perkin Elmer AB1 Prism 377 (Applied Biosystems, Foster City, CA). The nucleotide sequences were 100% identical to those of published cDNA or mRNA sequences for ChAT, NGF, and β-actin.

Synthesis of Specific cDNA Probes. Purified RT-PCR products (1 μl, ChAT, NGF or β-actin) were then used as templates in subsequent PCR reactions to synthesize ^{32}P-dCTP-labeled cDNA probes, using the procedure reported by Pan and Failla (22). Briefly, the PCR mix (40 μl) contained 1 μl purified template, 0.8 μl dATP (10 m*M*), 0.8 μl dGTP (10 m*M*), 0.8 μl dTTP, 4.0 μl 10X PCR buffer, 3.2 μl $MgCl_2$ (25 m*M*), 0.4 μl primers (20 μ*M*), 23.35 μl water, and 0.25 μl Taq DNA polymerase (5 U/μl), and 5 μl ^{32}P-dCTP (3000 Ci/mmol, Amersham Lifescience, Arlington Heights, IL). The PCR cycle conditions were 94°C for 1 min, 65°C for 1 min, and 72°C for 40 sec, and the reactions were conducted for 20 cycles, followed by a final extension at 72°C. The resulting ^{32}P-labeled cDNA probes were purified with Select G-50 STE columns before they were used in the following Northern analysis.

Northern Analysis. RNA samples prepared from the frontal cortex or hippocampus were subjected to Northern analysis as described by Pan and Failla (22). Briefly, total RNA samples (30 μg) were separated on 1.0% agarose gel by electrophoresis. Separated RNA was transferred to nylon membranes in 10X SSC at room temperature overnight (18 hr). The membranes were exposed to a UV-crosslinker to immobilize the transferred RNA, and then incubated with prehybridization solution (50% formamide, 5X SSPE, 5X Denhardt's solution, 1% SDS, and 10 μg/ml denatured Herring sperm DNA) at 42°C for 4 hr. Finally, the membranes were incubated with a specific PCR probe (ChAT, NGF, or β-actin) in hybridization solution (50% formamide, 5X SSPE, 1% SDS, and 10 μg/ml denatured Herring sperm DNA) at 42°C for 20 hr. After incubation, the membranes were washed and subjected to autoradiography and then PhosphorImager analyses, using Molecular Dynamics PhosphorImager 445 SI and ImageQuaNT software (Molecular Dynamics World Headquarters, Sunnyvale, CA). The optical density readings of ChAT and NGF were normalized with the optical density readings of β-actin obtained by reprobing the same membrane with a β-actin-specific probe. In addition to phosphorImager analyses, the membranes were subjected to autoradiography using Kodak BIOMAX MS film and intensifying screens at −70°C.

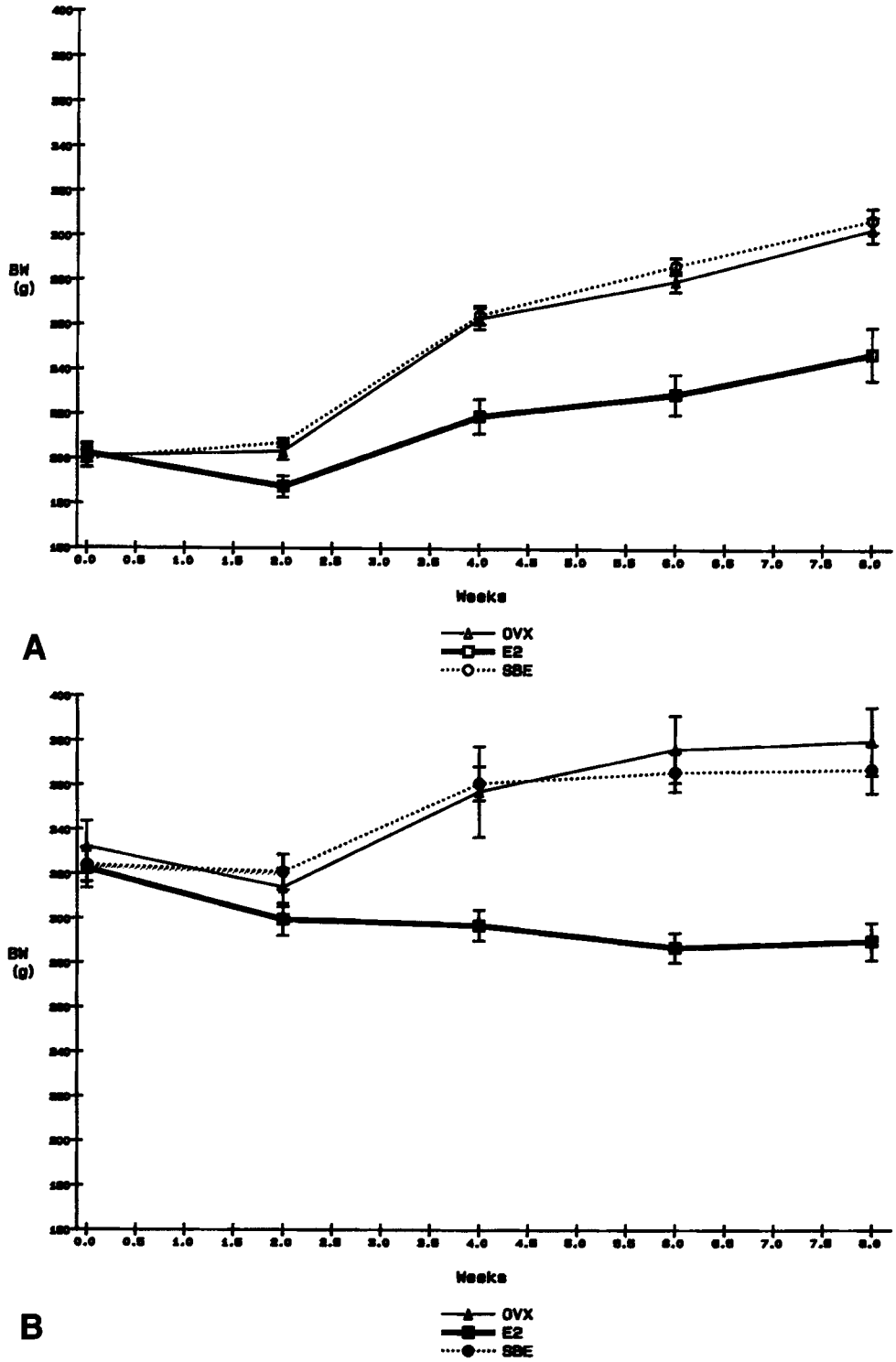

Figure 1. Effects of ovariectomy (OVX), oral micronized estradiol (E2), and soy phytoestrogens (SBE) on the body weights of (A) young and (B) retired breeder rats. Within each age category, five bilaterally ovariectomized rats were fed either a soy-free control diet (OVX), or the control diet with added estradiol (E2), or the control diet with added soy phytoestrogens (SBE) for 8 weeks, and the body weights were weighed every 2 weeks until necropsy. The results are means ± SEM. $n = 5$ per group.

Serum Estradiol and Phytoestrogen Analyses. Serum estradiol concentrations were measured from blood samples taken at necropsy at Yerkes Regional Primate Research Center Endocrinology Laboratory. This assay was modified from a commercially available RIA kit that uses a double-antibody technique (Diagnostic Products, Los Angeles, CA). Serum genistein, daidzein, and equol concentrations were measured at ESA, Inc. (Chelmsford, MA), using a protocol modified from a previous paper (23).

Body and Uterine Weights. The body weights of the rats were weighed with an electronic balance every 2 weeks until necropsy, and the uterus weights were measured with an electronic balance at necropsy.

Statistical Analyses. All analyses were done using BMDP Statistical Software, version 7.0 (Los Angeles, CA). Brain biomarkers (NGF and ChAT), serum estradiol, and uterine weight were analyzed by ANOVA. Body weight data were analyzed by repeated measure ANCOVA using

Table II. Serum Estradiol Concentrations and Uterus Weights (mean ± SEM) in Ovariectomized Young Adult and Retired Breeder Rats Treated with No Hormone Treatment (OVX), Oral Micronized Estradiol (E2), or Soy Phytoestrogens (SBE) for 8 weeks

Measurement	Age group	OVX	E2	SBE	ANOVA p-value
Estradiol (pg/ml)	Young adults*	$9.15^a \pm 1.56$	$24.75^b \pm 2.61$	$4.09^a \pm 1.04$	<0.0001
	Retired breeders*	$7.58^a \pm 1.25$	$29.58^b \pm 3.70$	$7.25^a \pm 1.76$	<0.0001
Uterus weight (g)	Young adults*	$0.106^a \pm 0.007$	$0.383^b \pm 0.019$	$0.106^a \pm 0.012$	<0.0001
	Retired breeders*	$0.156^a \pm 0.010$	$0.428^b \pm 0.021$	$0.149^a \pm 0.006$	<0.0001

* Group means with different letters within each age group are significantly different using Student-Newman-Keuls multiple-range test to adjust for multiple comparisons.

pretreatment body weight as the covariate. Between-group comparisons were done adjusting for multiple comparison by the Student-Newman-Keuls multiple range test. Because there were differences in the effects of treatment on some of the variables in the two age groups, the data were analyzed separately for the young animals and retired breeders.

Results

Body Weights, Uterine Weights, Serum E2, and Phytoestrogen Concentrations. All groups except the E2-treated retired breeder rats gained weight during the 8 weeks of treatment (Fig. 1). The E2-treated groups had significantly lower body weights ($P < 0.0001$) than either OVX rats or SBE-treated rats within their age category. The body weights of the OVX and SBE groups within each age category were not different. Uterine weights and serum estradiol concentrations measured at necropsy are shown in Table II. As expected, serum estradiol concentrations and uterine weight were significantly higher in the E2 groups ($P < 0.0001$). The OVX and SBE groups were not different from each other for either serum estradiol concentrations or uterine weights. The concentrations of genistein, daidzein, and equol of a pool of serum samples taken from five SBE-treated young rats were 394.1, 175.0, and 815.6 n*M*, respectively. Similarly, 343.8 n*M* genistein, 157.3 n*M* daidzein, and 872.5 n*M* equol were measured in the pooled serum samples taken from five SBE-treated retired breeder rats. These data indicated that oral soy phytoestrogens are readily absorbed into the blood by female rats.

NGF mRNA in the Frontal Cortex and Hippocampus. Specific RT-PCR product of NGF was detected in the frontal cortex and hippocampus of young adult and retired breeder rats in all three treatment groups using RT-PCR (data not shown), showing the presence of NGF mRNA in these two brain regions. Subsequently, Northern analysis was used to determine the relative levels of NGF mRNA, using total RNA isolated from the frontal cortex and hippocampus of animals. PhosphorImager (Molecular Dynamics World Headquarters, Sunnyvale, CA) analysis showed that the NGF mRNA level in the hippocampus was significantly higher in young E2 rats than in young OVX rats. The NGF mRNA level in the hippocampus of SBE rats was intermediate, but not significantly different from either E2 rats or OVX rats (Fig. 2). Northern analyses of total RNA failed to detect NGF mRNA in the frontal cortex of both young and retired breeder rats and in the hippocampus of retired breeder rats.

ChAT mRNA in the Frontal Cortex and Hippocampus. Specific RT-PCR product of ChAT was detected in the frontal cortex and hippocampus of young adult and retired breeder rats in all three treatment groups using RT-PCR (data not shown), indicating the presence of ChAT mRNA in these two brain regions. PhosphorImager analysis of Northern blot membranes showed that ChAT mRNA levels in the frontal cortex were not affected by estrogen replacement, nor by phytoestrogen supplements in young ovariectomized rats (Fig. 3). Similarly, ChAT mRNA levels in the hippocampus did not differ among these three treatments in young rats (Fig. 4). In contrast, 2 months after OVX, ChAT mRNA levels were significantly higher in the frontal cortex of E2-treated (34%) and SBE-treated (38%) retired breeder rats compared with OVX retired breeder rats (Fig. 3). However, ChAT mRNA levels in the hippocampus were not different among OVX, E2-treated, and SBE-treated retired breeders (Fig. 4).

Discussion

Our data showed that oral soy phytoestrogens, at the relatively high dose used in this experiment, had no estrogen agonist effect on the uterus as indicated by the significantly lower uterine weight in SBE and OVX rats compared to that of E2 rats, which further confirmed previous reports that soy phytoestogens have no estrogenic effects on the uterus (17). Specific RT-PCR product of NGF was observed in the frontal cortex and hippocampus of both young adult and retired breeder rats in all treatment groups. This was in agreement with previous reports concerning NGF mRNA in these brain regions (5, 10, 24). Northern analysis showed that 2 months of estrogen deficiency induced by ovariectomy resulted in lower NGF mRNA levels, and oral estrogen supplement for 2 months resulted in significantly higher NGF mRNA levels in the hippocampus of young adult rats, which was consistent with the observation by Singh *et al.* (10). Gibbs *et al.* (2) showed that compared to ovariectomized controls, NGF mRNA levels in the hippocampus of adult Sprague-Dawley rats were reduced by short-term estradiol replacement (2

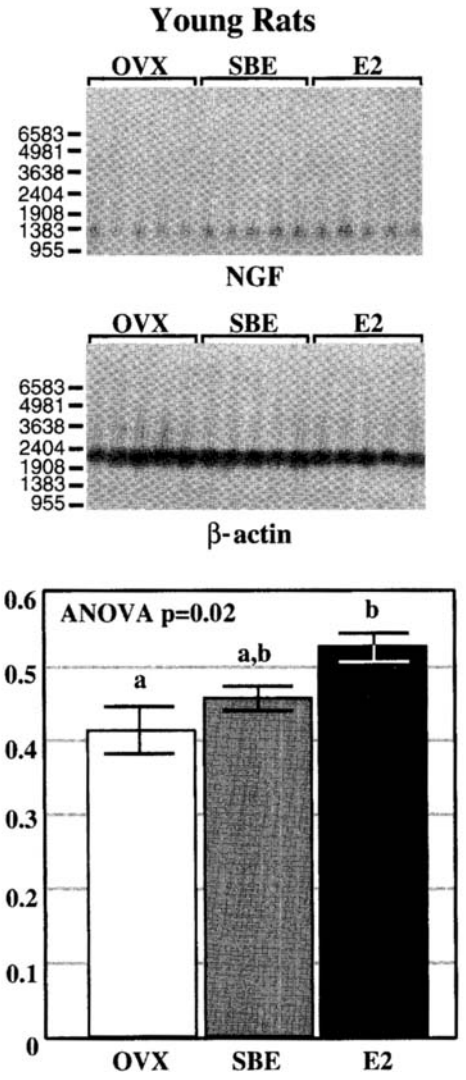

Figure 2. Effects of estradiol (E2) and soy phytoestrogens (SBE) on nerve growth factor (NGF) mRNA in the hippocampus of young rats. Five bilaterally ovariectomized rats were fed either a soy-free control diet (OVX), or the control diet with added estradiol (E2), or the control diet with added soy phytoestrogens (SBE) for 8 weeks. Total RNA samples (30 μg), which were isolated from tissues of the hippocampus of young rats, were subjected to Northern and PhosphorImager Analyses as described in "Materials and Methods." The results are means ± SEM. $n = 5$ per group.

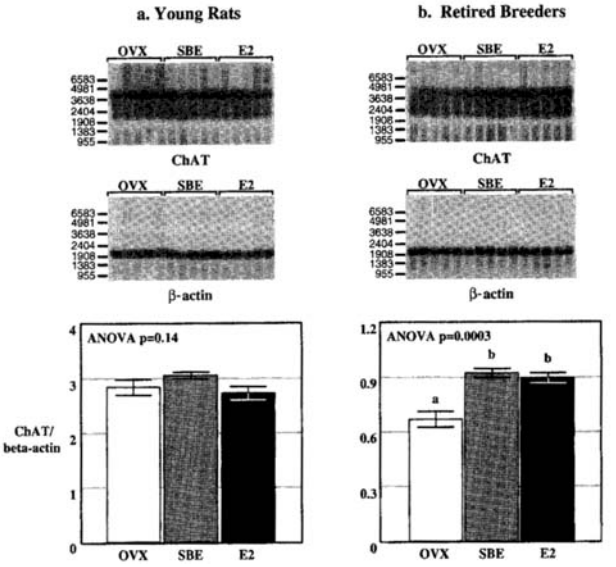

Figure 3. Effects of estradiol and soy phytoestrogens on choline acetyltransferase (ChAT) mRNA in the frontal cortex of young and retired breeder rats. Within each age category, five bilaterally ovariectomized rats were fed either a soy-free control diet (OVX), or the control diet with added estradiol (E2), or the control diet with added soy phytoestrogens (SBE) for 8 weeks. Total RNA samples (30 μg), which were isolated from tissues of the frontal cortex of young and retired breeder rats, were subjected to Northern and PhosphorImager Analyses as described in "Materials and Methods." The results are means ± SEM. $n = 5$ per group.

days and 1 week) and were not affected by 2 weeks of estradiol replacement. Recently, Gibbs (25) showed that NGF mRNA in the hippocampus was not affected by estrous cycle and that 24, 53, and 72 hr after a single injection (sc) of 10 μg 17β-estradiol, NGF mRNA levels were not affected compared to ovariectomized controls. These data suggest that NGF mRNA levels are not affected by relatively short-term estrogen replacement. On the other hand, our data suggest that 8-week estrogen deficiency induced by ovariectomy resulted in lower levels of NGF mRNA in the hippocampus of young rats compared to E2-treated animals. Soy phytoestrogens appear to have a marginal effect on NGF mRNA in the hippocampus of young adult rats, which merits further investigation and confirmation. Even though specific RT-PCR product of NGF was observed in these two brain regions regardless of age and estrogen status, NGF mRNA was not detected by Northern analysis of total RNA isolated from the frontal cortex of either young or retired breeder rats or the hippocampus of retired breeder rats, suggesting that NGF mRNA levels are higher in the hippocampus than in the frontal cortex of young adult rats, and that NGF mRNA levels are decreased in the brains of older animals. In fact, Larkfors *et al.* (24) reported that NGF mRNA in the cerebral cortex, hippocampus, basal forebrain, and hypothalamus was reduced by 50% in aged brain (28 months) compared to the adult brain (6 months) in Fischer 344 rats. These data suggest that NGF mRNA decreases with ageing in these brain regions in rats. More sensitive analytical tools, such as Northern analysis using poly(A)$^+$ RNA, RNase Protection Assay, or quantitative RT-PCR, can be used to determine whether estrogen deficiency decreases NGF mRNA in the frontal cortex and hippocampus in aged brain and whether estrogen and soy phytoestrogens upregulate NGF mRNA in these brain regions in aged rats under the condition of surgery-induced estrogen deficiency.

RT-PCR analysis indicated that ChAT mRNA is present in the frontal cortex and hippocampus of both young and retired breeder rats, which confirms previous reports of the presence of ChAT mRNA in these two regions (11, 14). Northern analysis of total RNA isolated from the frontal cortex and hippocampus of both young and retired breeder rats revealed a 4.4-kb band, which was consistent with the reported size of ChAT mRNA in rats (26). PhosphorImager analysis of Northern blot membranes indicated that ChAT mRNA levels in the frontal cortex and hippocampus of

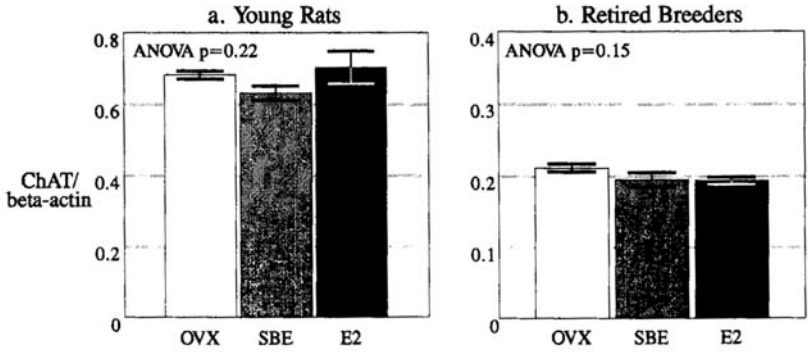

Figure 4. Effects of estradiol and soy phytoestrogens on choline acetyltransferase (ChAT) mRNA in the hippocampus of young and retired breeder rats. Within each age category, five bilaterally ovariectomized rats were fed either a soy-free control diet (OVX), or the control diet with added estradiol (E2), or the control diet with added soy phytoestrogens (SBE) for 8 weeks. Total RNA samples (30 μg), which were isolated from tissues of the hippocampus of young and retired breeder rats, were subjected to Northern and PhosphorImager Analyses as described in "Materials and Methods." The results are means ± SEM. $n = 5$ per group.

young adult rats were not significantly different among treatment groups. In contrast, ChAT mRNA levels were significantly higher in the frontal cortex of E2 and SBE rats compared to OVX group but were not significantly different in the hippocampus among treatment groups in the retired breeder rats. These data may suggest that an 8-week treatment is too short to detect significant changes in ChAT mRNA in the frontal cortex and hippocampus of young rats and in the hippocampus of retired breeders. Another possibility is that different regions of the brain are more or less susceptible to estrogen status (lack of effect in the hippocampus of young and retired breeder rats) and that young rats are less sensitive to estrogen deficiency/replacement (no effect in either the frontal cortex or hippocampus). Singh *et al.* (2) reported that long-term ovariectomy reduced ChAT activity in the frontal cortex of rats by 50% and had little impact on ChAT activity in the hippocampus, suggesting that the frontal cortex is more susceptible to E2 deficiency than the hippocampus. Singh *et al.* (2) also reported that short-term E2 treatment of young rats had no impact on ChAT activity in the frontal cortex, but significantly increased ChAT activity in the hippocampus (18%).

Others reported that ChAT activity in the frontal cortex and hippocampus was decreased during surgery-induced estrogen deficiency and could be reversed by estrogen replacement (2, 9, 10). These data suggested that ChAT protein in these brain regions is affected by estrogen status. In addition, Berger and Gaspar (27) reported that cholinergic projection from the nucleus basalis magnocellularis (NBM) makes about a 60%–70% contribution to the cerebral cortex ChAT activity, and that the rest (30%–40%) of cerebral cortex ChAT comes from cortex cholinergic neurons and cholinergic projections from the brain stem. Destroying the afferent cholinergic connections of the hippocampus reduced the ChAT activity, rather than eliminated it in the hippocampus (28). These data showed that local ChAT mRNA also makes a contribution to ChAT activity in these regions. Therefore, it is important to investigate how estrogen deficiency and replacement affect local cholinergic neurons. Our data indicated that local ChAT mRNA in the frontal cortex of retired breeder rats was affected by estrogen status, suggesting possible involvement of cortex cholinergic neurons in maintaining cortex ChAT activity, which is positively associated with cognition.

There is no previous information concerning the effects of soy phytoestrogens on ChAT mRNA levels in the brain. Our data showed that soy phytoestrogens are very effective in upregulating ChAT mRNA in the frontal cortex of retired breeder rats under the condition of estrogen deficiency, suggesting that soy phytoestrogens may be an effective estrogen agonist in regulating ChAT mRNA. Faber and Hughes (29) reported that a high dose of genistein functioned as an estrogen agonist in regulating LH secretion and the volume of the sexually dimorphic nucleus of the preoptic area in ovariectomized female rats, indicating that genistein is at least an estrogen agonist in regulating classic estrogen-responsive brain regions. The mechanisms by which estrogen exerts its protective effect on cognitive function are still not clear. It is plausible that estrogen, as a member of the steroid hormone family, may, at least partially, function through its receptors (30, 31). Two subtypes of estrogen receptor (ERα and ERβ) have been reported (32–34); ERβ is reported to be the major subtype of ER in the brain (35). Interestingly, genistein's binding affinity to ERβ is only three times lower than the potent 17β-estradiol (35), which provides molecular basis for soy phytoestrogens, or at least genistein, to interact with ERβ and induce estrogenic effects. Further studies are needed to confirm our observation, to understand the mechanisms behind it, and to determine the binding affinity of other soy phytoestrogens to ER.

The authors acknowledge the contributions of Professors Bill Bullock and Michael Adams, Dr. Claude L. Hughes Jr., and the financial support of Protein Technologies International, Inc., St. Louis, MO.

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