

# Neuroprotective and Neurotrophic Efficacy of Phytoestrogens in Cultured Hippocampal Neurons

LIXIA ZHAO, QI CHEN, AND ROBERTA DIAZ BRINTON<sup>1</sup>

Department of Molecular Pharmacology and Toxicology and Neuroscience Program, University of Southern California, Pharmaceutical Sciences Center, Los Angeles, California 90089

Epidemiological data from retrospective and case-control studies have indicated that estrogen replacement therapy (ERT) can decrease the risk of developing Alzheimer's disease. In addition, ERT has been found to promote cellular correlates of memory and to promote neuronal survival both *in vivo* and *in vitro*. Phytoestrogens have been proposed as potential alternatives to ERT. To determine whether phytoestrogens exert estrogen agonist effect in neural tissue, investigations of neuroprotective and neurotrophic efficacy of phytoestrogens were conducted. Six phytoestrogens, genistein, genistin, daidzein, daidzin, formononetin, and equol, were tested for their neuroprotective efficacy against two toxic insults, glutamate excitotoxicity and  $\beta$ -amyloid<sub>25-35</sub>. Neuronal membrane damage was quantitatively measured by lactate dehydrogenase (LDH) release, and neuronal mitochondrial viability was determined by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay. Results of these studies demonstrated that all phytoestrogens induced a modest but significant reduction in LDH release following exposure to glutamate and  $\beta$ -amyloid<sub>25-35</sub>. In contrast, none of phytoestrogens induced a significant increase in reduced MTT levels, which occurred in the presence of a full estrogen agonist, 17 $\beta$ -estradiol. Analysis of the neurotrophic potential of genistein and daidzein, two phytoestrogens that exerted a significant reduction in LDH release, demonstrated that neither of these molecules promoted hippocampal neuron process outgrowth. Results of these analyses indicate that although phytoestrogens exert a neuroprotective effect at the plasma membrane, they do not sustain neuron mitochondrial viability nor do they induce cellular correlates of memory as neurite outgrowth and synaptogenesis are putative mechanisms of memory. Data derived from these investigations would predict that phytoestrogens could exert some neuroprotective effects analogous to that of antioxidants, but that these mol-

ecules are not functional equivalents to endogenously active 17 $\beta$ -estradiol or to estrogen replacement formulations and, therefore, would raise the concern that they may not reduce the risk of Alzheimer's disease or sustain memory function in postmenopausal women.

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Phytoestrogens are plant-derived molecules that structurally resemble endogenous estrogens containing a diphenolic chemical structure that can directly bind to estrogen receptors (ER) to regulate gene expression mediated by estrogen response element (1). Select phytoestrogens have been found to exhibit some estrogen agonist-like properties (2, 3). However, phytoestrogens also act as partial estrogen receptor antagonists (4). Cellular mechanisms activated by phytoestrogens are diverse and appear to be concentration dependent (5). For example, at low concentrations, phytoestrogens can induce proliferation of ER-positive MCF-7 cells, but not of ER-negative MDA-MB-231 cells (5). Conversely, several studies have shown anti-proliferative effects of phytoestrogens on human breast cancer cell lines and in animal experiments. High concentrations of phytoestrogens (comparable with those achieved in plasma following consistent soy consumption) can significantly inhibit proliferation of human breast carcinoma cell growth (5-8).

One mechanism proposed to account for the inhibitory action of phytoestrogens is competitive inhibition with endogenous estrogens through binding to the ERs. Indeed, some phytoestrogens competitively suppress the binding of 17 $\beta$ -estradiol to ERs in human mammary tumor tissue (9, 10). Coumestrol has been demonstrated to antagonize neuroendocrine actions of estrogen in the brain and pituitary via ER $_{\alpha}$  (11). Recent data indicate that phytoestrogens exhibit a greater affinity for ER $_{\beta}$  relative to ER $_{\alpha}$  (9). This finding is of interest because ER $_{\beta}$  has a higher level of expression than that of ER $_{\alpha}$  in brain regions critical to memory function and vulnerable to Alzheimer's disease such as the basal fore-

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<sup>1</sup> To whom requests for reprints should be addressed at Department of Molecular Pharmacology and Toxicology, School of Pharmacy, University of Southern California, 1985 Zonal Avenue, Los Angeles, CA 90089-9121. E-mail: rbrinton@hsc.usc.edu

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brain, hippocampus, and cerebral cortex (3). Other potential mechanisms of phytoestrogen action are through tyrosine kinase inhibition, DNA topoisomerase inhibition, inhibition of aromatase function, and stimulation of sex hormone-binding globulin in the liver, thereby reducing free, biologically active estradiol in the plasma and by inducing antioxidant properties (12–16). Because of their mixed agonist/antagonist ER profile, phytoestrogens have received considerable attention as potential alternatives to estrogen.

Increasing evidence suggests that a loss in estrogen can increase a women's risk of developing Alzheimer's disease (17, 18). Epidemiological data from retrospective and case-control studies have indicated that postmenopausal women receiving estrogen replacement therapy (ERT) exhibit a decreased risk of developing Alzheimer's disease (17, 19–24). Furthermore, *in vitro* studies have demonstrated neuroprotective effects of estrogens against glutamate- and  $\beta$ -amyloid-induced neurotoxicity (25–28). Our laboratory and others also reported that 17 $\beta$ -estradiol promoted neuronal process outgrowth both *in vivo* and *in vitro* (27–34).

Despite the beneficial effects of ERT on cognition, estrogen use in postmenopausal women is associated with increased risks of uterine cancer and potentially an increase in neoplasms of the breast (35), especially after long-term use (36, 37). In contrast, several reports indicate that phytoestrogens do not promote neoplasms of the breast and uterus, but instead reduce the risk of developing several types of cancer, most notably breast cancer (38–44). Phytoestrogen-associated risk reduction of breast cancer is of critical significance not only for the potential of reducing the incidence of breast cancer, but also because the specter of breast cancer has a significant and pervasive influence on health choices that impact other disease risks. The fear of breast cancer is the major negative factor women cite for deciding against using ERT (35).

Although the evidence concerning phytoestrogens and reduction of breast cancer risk appears encouraging, the evidence concerning protection against Alzheimer's disease is lacking. Most women spend one-third to one-half of their lifetime in the menopausal state. Currently, there are over 470 million women aged 50-plus worldwide, and 30% of those will live to the age of 80 (<http://www.menopause.org/>; North American Menopause Society, 2001). Given that age remains the greatest risk factor for developing Alzheimer's disease, a question of increasing clinical importance is whether phytoestrogens are indeed a safe and effective alternative to ERT for the maintenance of cognitive function and prevention of Alzheimer's disease (28). To address this question, we investigated the efficacy of phytoestrogens to protect against damage induced by neurotoxic levels of  $\beta$ -amyloid and glutamate using lactate dehydrogenase (LDH) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assays. LDH is a stable cytoplasmic enzyme present in all cells and is rapidly released into the cell culture supernatant when the plasma membrane is damaged, thus, it can be used as a reliable biochemical index for

neuronal plasma membrane damage. MTT is an index of mitochondrial viability because it is reduced by metabolically active mitochondria (57–60). Furthermore, because the hippocampus is critical for the early phases of learning and memory, is affected early in the course of Alzheimer's disease, and hippocampal neuronal neurite outgrowth has been suggested to be a putative mechanism for memory (28), we sought to determine whether select phytoestrogens induced hippocampal neuron process outgrowth as a marker of phytoestrogen regulation of memory mechanisms.

## Materials and Methods

**Neuronal Culture.** Use of animals has been approved by the Institutional Animal Care and Use Committee (IACUC) at University of Southern California (Protocol No. 9052). Primary cultures of hippocampal neurons were performed as described in Brinton (27). Briefly, hippocampi were dissected from the brains of embryonic Day 18 (E18 d) rat fetuses, treated with 0.02% trypsin in Hanks' balanced salt solution (137 mM NaCl, 5.4 mM KCl, 0.4 mM  $\text{KH}_2\text{PO}_4$ , 0.34 mM  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ , 10 mM glucose, and 10 mM HEPES) for 5 min at 37°C and dissociated by repeated passage through a series of fire-polished constricted Pasteur pipettes. For morphological analyses, between 20,000 and 40,000 cells were seeded onto polylysine-coated (10  $\mu\text{g}/\text{ml}$ ) 22-mm coverslips, whereas cultures used for biochemical (LDH and MTT) analyses were plated onto 0.1% polyethylenimine coated 96-well culture plates (VWR) at a density of  $10^5$  neurons/ml. Nerve cells were grown in Neurobasal medium (NBM; Gibco/Life Technologies, St. Petersburg, FL) supplemented with B27, 5 U/ml penicillin, 5  $\mu\text{g}/\text{ml}$  streptomycin, 0.5 mM glutamine, and 25  $\mu\text{M}$  glutamate, at 37°C in 10%  $\text{CO}_2$ . Cultures grown in serum-free NBM yield approximately 99.5% neurons and 0.5% glia. Microscopically, glial cells were not apparent in hippocampal cultures at the times these cultures were used for experimental analyses.

**Morphological Analysis.** Nerve cells attached to polylysine-coated coverslips were removed from the culture dish and rapidly mounted into a recording chamber. The volume of the chamber was 200  $\mu\text{l}$ , and pH was maintained at 7.4. Videomicroscopic recording of nerve cells was accomplished using a Dage-MTI camera equipped with a Newvicon tube linked to an Olympus BH-2 microscope and a Panasonic time-lapse video recorder (Model AG-6050). Recordings were made using phase contrast optics with a  $\times 40$  objective and a 1.50 multiplier with 100 W tungsten source passed through a green filter. Nerve cell recordings were conducted following 24 hr of exposure to test substances. Selection of nerve cells for analysis was random, and all recording and morphological analyses were conducted blind to the experimental condition. Morphological analysis was achieved using a BioQuant Image Analysis system designed for quantitative analysis of cellular morphological features. Cell size was controlled by selecting an equal number of cells from each coverslip that fell within

three size categories: small, medium, and large. Cell size was determined by the area of the field encompassed by the length of extensions. If a cell encompassed one-fourth of the monitor field, it was categorized as small, one-half of the field was categorized as medium, and the entire monitor field or required multiple fields for analysis was categorized as large. Number of neurites was defined as the number of extensions greater than 50  $\mu\text{m}$  in length emanating directly from the cell body. Neurite length represents the summation of the length of all neurites/neuron. Branches were operationally defined as any extension that exceeded 10  $\mu\text{m}$  in length and occurred along the shaft of the neurite. Branches that occurred as second or third order processes were not included in this measure. Branch length represents the summation of the length of all branches present on an individual neuron. The number of bifurcation points represents the total number of points at which branches extend from the neuritic shafts plus those points at which branches extend from other branches for an entire neuron. Microspikes were defined as processes emanating from either neurites or branches that measured less than 10  $\mu\text{m}$ . Statistically significant differences were determined by a one-way analysis of variance (ANOVA) followed by a Newman-Keuls *post hoc* analysis for individual differences.

#### **Efflux Assay of LDH and Reduction Assay of**

**MTT.** Neurotoxins utilized included 0.1 mM glutamate (Sigma, St. Louis, MO) and 8  $\mu\text{g/ml}$   $\beta$ -amyloid peptide<sub>25-35</sub> ( $\text{A}\beta_{25-35}$ ; Lot number B01200; Bachem, Torrance, CA). Glutamate solution was prepared prior to each experiment, whereas  $\text{A}\beta_{25-35}$  was dissolved in sterile distilled water at a concentration of 1 mg/ml as a stock solution. This stock was aliquoted and stored at  $-20^\circ\text{C}$ . Neuronal cultures grown in 96-well plates were pretreated with estrogens at varying concentrations for 48 hr prior to exposure to glutamate at room temperature for 5–10 min in HEPES buffer containing (in millimoles) 100 NaCl, 2.0 KCl, 2.5  $\text{CaCl}_2$ , 1.0  $\text{MgSO}_4$ , 1.0  $\text{NaH}_2\text{PO}_4$ , 4.2  $\text{NaHCO}_3$ , 12.5 HEPES, and 10.0 glucose, or to  $\beta$ -amyloid peptide<sub>25-35</sub> for 24 hr in NBM media. Following exposure to the neurotoxins, cultures were washed two times with  $37^\circ\text{C}$  HEPES buffer and replaced with fresh NBM media. Cultures were then returned to the incubator, and assessment of LDH release in the media was conducted 24 hr following exposure to the neurotoxins. LDH release into the culture media was measured using a Cytotoxicity Detection kit from Boehringer Mannheim Biochemicals (Indianapolis, IN) and absorption was read at 490 nm. MTT was then added to the culture at a final concentration of 5 mg/ml for 4 hr and the reaction was stopped by the addition of 1 mM HCl in 10% SDS. Formazan crystals were dissolved overnight and absorption was read at 595 nm. In the 96-well plates, each condition has 6–12 replicate wells. This number of replicates allows us to generate a mean and SEM from each experiment. These data are the basis of that reported for “one representative experiment.” Each experiment is conducted a minimum of three to four times. Statistically significant differences between conditions from

one representative experiment were determined by a one-way ANOVA followed by a Newman-Keuls *post hoc* analysis for individual comparisons.

**Glutamate Exposure.** One-week-old neuron cultures were pretreated with estrogens for 2 days and were then exposed to 0.1 mM glutamate for 5–10 min at room temperature in HEPES buffer containing (in millimoles) 100 NaCl, 2.0 KCl, 2.5  $\text{CaCl}_2$ , 1.0  $\text{MgSO}_4$ , 1.0  $\text{NaH}_2\text{PO}_4$ , 4.2  $\text{NaHCO}_3$ , 12.5 HEPES, and 10.0 glucose. Immediately following glutamate exposure, cultures were washed two times with HEPES buffer and replaced with fresh NBM. Cultures were returned to the culture incubator and allowed to incubate for 24 hr prior to LDH and MTT measurements.

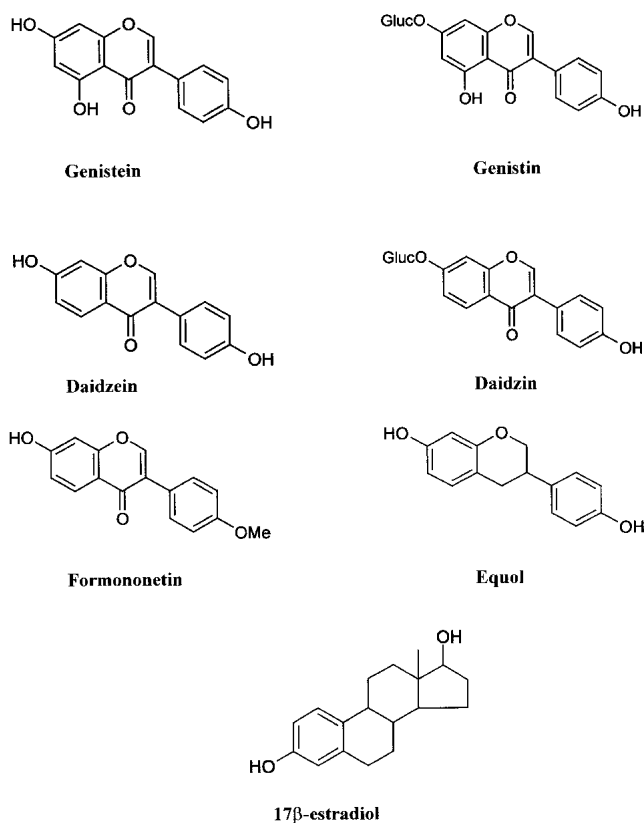
**$\beta$ -Amyloid<sub>25-35</sub> ( $\text{A}\beta_{25-35}$ ) Exposure.**  $\text{A}\beta_{25-35}$  (Lot number B01200), purchased from Bachem, was dissolved in sterile distilled water at a concentration of 1  $\mu\text{g/ml}$  as a stock solution. This stock was aliquoted and stored at  $-20^\circ\text{C}$ . One-week-old neuron cultures were pretreated with varying concentrations of estrogens for 2 days in NBM. Exposure to  $\text{A}\beta_{25-35}$  followed the procedure described by Behl *et al.* (45). After 2 days of culture with estrogens, NBM containing  $\text{A}\beta_{25-35}$  alone or  $\text{A}\beta_{25-35}$  plus varying concentrations of estrogens were prepared, and cultures were incubated in test substances for 24 hr at  $37^\circ\text{C}$ . After 24 hr of exposure to  $\text{A}\beta_{25-35}$ , culture medium was exchanged for  $\text{A}\beta_{25-35}$ -free medium with estrogens. Cultures were returned to the incubator and allowed to incubate for 24 hr prior to LDH and MTT measurements.

**Chemicals.**  $\beta$ -Amyloid peptide<sub>25-35</sub> was obtained from Bachem; phytoestrogens, genistein, genistin, daidzein, daidzin, formononetin, and equol were purchased from Indofine Chemical Company (Somerville, NJ); 17 $\beta$ -estradiol and the MTT powder were purchased from Sigma, and the LDH assay kit was purchased from Boehringer Mannheim Biochemicals.

## **Results**

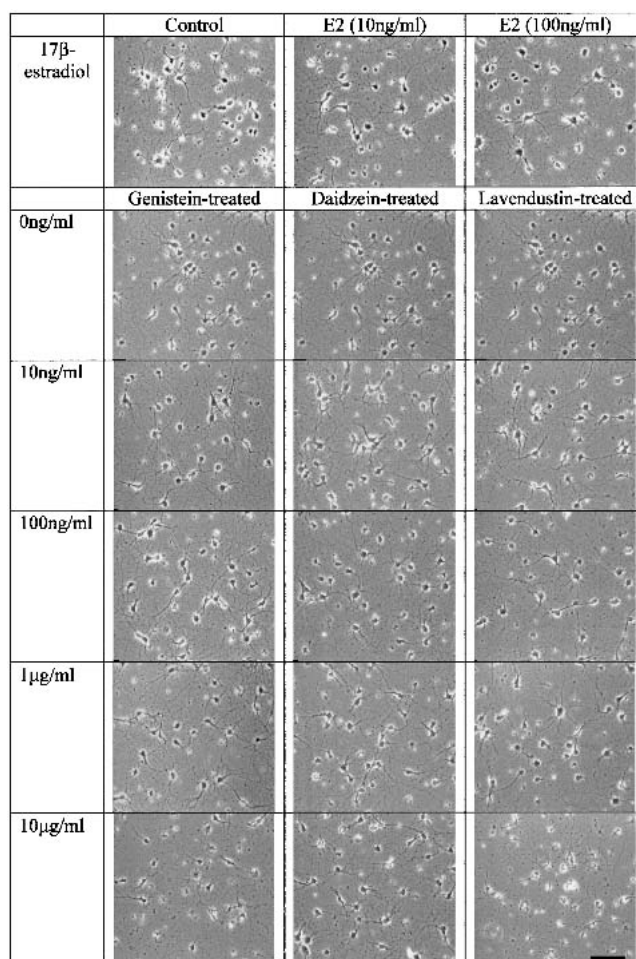
The phytoestrogens, genistein, genistin, daidzein, daidzin, formononetin, and equol were selected based on their presence in dietary sources (1). Phytoestrogen chemical structures relative to that of 17 $\beta$ -estradiol are shown in Figure 1.

**Effect of Phytoestrogens on Hippocampal Neuron Morphology.** To determine an effective concentration range for neuroprotection and neurotrophism experiments, we first investigated the impact of varying concentrations of genistein and daidzein on hippocampal neuron morphological integrity and survival. Genistein and daidzein are the two most common phytoestrogens contained in soybeans (46), and they are structurally related to the other phytoestrogens used in the study in that they differ only by a hydroxyl group (Fig. 1). Because genistein can inhibit tyrosine kinase activity and has been broadly used as a tyrosine kinase inhibitor in a variety of studies, we used lavendustin A, another tyrosine kinase inhibitor, as a control to discriminate estrogenic activities and tyrosine



**Figure 1.** Comparison of the chemical structures of phytoestrogens used in the current study with that of 17β-estradiol.

kinase inhibitor activities of genistein. Hippocampal neurons derived from E18-d rat fetuses were cultured in Neurobasal medium for 7 days prior to treatment with genistein, daidzein, and lavendustin A at 10 ng/ml, 100 ng/ml, 1 μg/ml, and 10 μg/ml concentrations for 48 hr. Because 17β-estradiol was used as a positive control in the following neuroprotective and neurotrophic analyses, images of neurons treated with 10 and 100 ng/ml E2 are also shown. Following 48 hr exposure, videomicroscopy was performed. Upon microscopic inspection, control cultures exhibited markers of viability; neurons with smooth dark cell bodies, phase bright halos, and extensive neuronal extensions (Fig. 2). Neurons treated with 10 and 100 ng/ml E2 showed healthy dark cell bodies with a phase bright halo, and they exhibited more clearly defined neuronal processes than control cells. Neurons treated with ≤1 μg/ml genistein, daidzein, or lavendustin A exhibited no morphological signs of toxicity and were indistinguishable from control neurons (Fig. 2). Cultures treated with 10 μg/ml genistein or lavendustin A, however, exhibited indicators of toxicity in that the cells exhibited shrunken cell bodies, collapsed cell membranes, and a decrement in the number of neuronal processes, whereas at the same concentration, daidzein did not induce morphological indicators of toxicity (Fig. 2). These data provided us with a concentration range, 10 ng/ml–1 μg/ml, that was below a toxic threshold and thereafter was used for subsequent experiments.



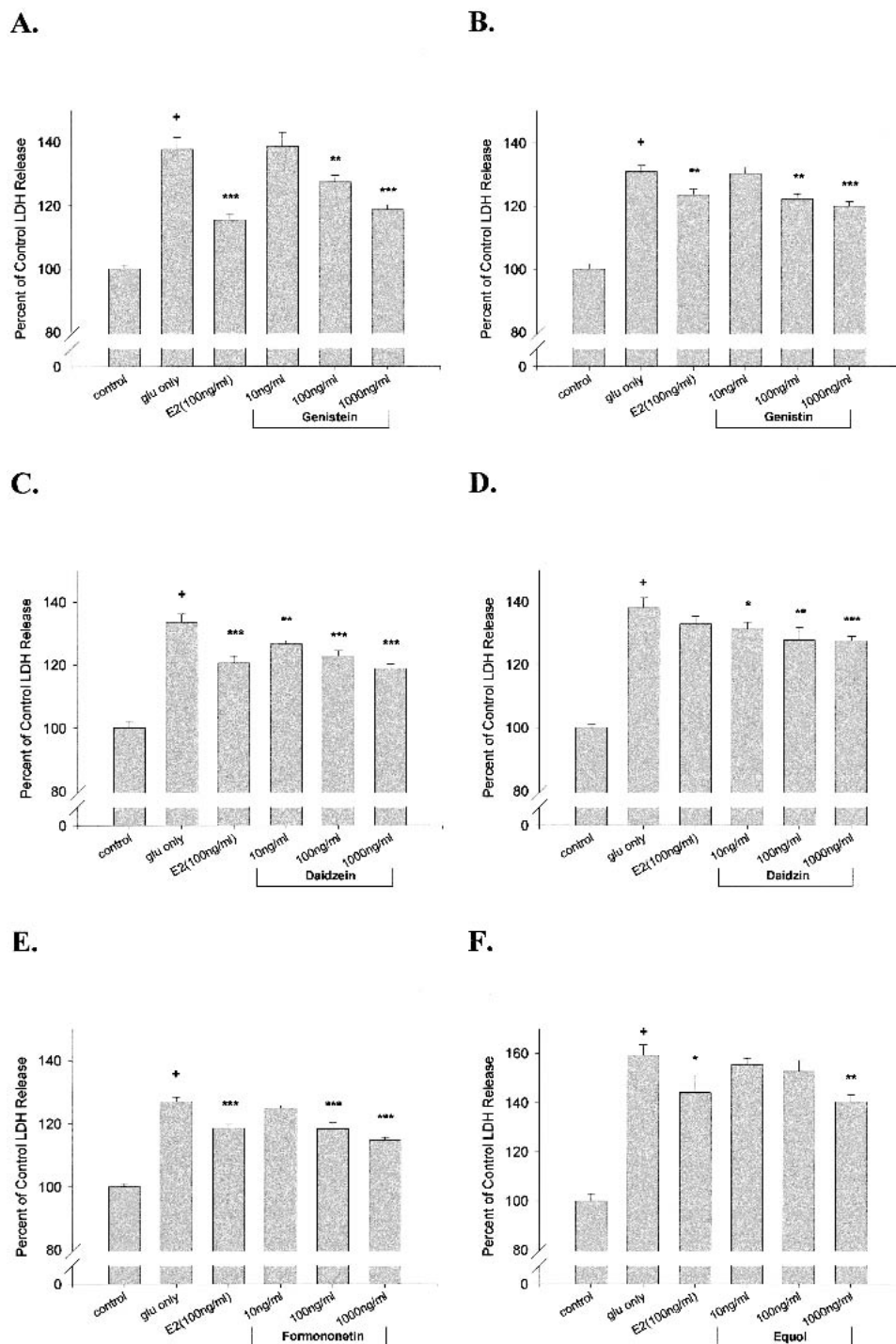
**Figure 2.** Effect of 17β-estradiol (E2), genistein, daidzein, and lavendustin on hippocampal neurons in culture. Neurons were cultured in NeuroBasal media for 7 days and treated with E2 at 10 and 100 ng/ml, and genistein, daidzein, and lavendustin at 10 ng/ml, 100 ng/ml, 1 μg/ml, and 10 μg/ml concentrations for 2 days. Images were video-recorded using phase-contrast microscopy. Hippocampal neurons under control condition appear healthy with large dark cell bodies and a phase bright halo and abundant clearly defined neuronal processes. Neurons treated with 17β-estradiol at 10 and 100 ng/ml showed healthy dark cell bodies and a phase bright halo, and they exhibited more clearly defined neuronal processes than control cells. Genistein, daidzein, and lavendustin at concentrations ≤1 μg/ml showed no morphological markers of toxicity, similar to control neurons. However, genistein and lavendustin at 10 μg/ml induced morphological markers of toxicity as manifested by shrunken cell bodies and a loss of process integrity. At the same concentration, daidzein did not exert a toxic effect in the neuronal culture significantly. Scale bar = 100 μm.

**Neuroprotective Efficacy of Phytoestrogens against Excitotoxic Glutamate.** The neuroprotective efficacy of phytoestrogens, genistein, genistin, daidzein, daidzin, formononetin, and equol against glutamate-induced neurotoxicity was assessed using LDH release as an indicator of plasma membrane damage and MTT reduction by viable mitochondria as an indicator of neuronal viability. Hippocampal neurons derived from E18-d rat fetuses were cultured in Neurobasal medium for 7 days and treated with test phytoestrogens at 10 ng/ml, 100 ng/ml, and 1 μg/ml for 48 hr prior to 100 μM glutamate exposure for 10 min.

Within the same experiment, 100 ng/ml 17 $\beta$ -estradiol was used as a positive control. Exposure to excitotoxic concentration of glutamate induced a significantly higher LDH release than control (ranging from 30% to 60%; Fig. 3). A significant reduction in glutamate-induced LDH release occurred at 100 ng/ml of 17 $\beta$ -estradiol. The magnitude of the 17 $\beta$ -estradiol-induced reduction in glutamate-induced toxicity ranged from 10% to 15%. Similar to 17 $\beta$ -estradiol, genistein, genistin, daidzein, daidzin, formononetin, and equol all showed modest but significant neuroprotection

against glutamate-induced LDH release. The magnitude of phytoestrogen-induced reduction in LDH release ranged from 8% to 15% (Fig. 3). Neither 17 $\beta$ -estradiol nor any of the test phytoestrogens alone had effect on LDH release by hippocampal neurons (data not shown).

Determination of phytoestrogen exposure on neuronal viability indicated that excitotoxic glutamate significantly reduced neuronal MTT levels (30%–40% reduction relative to control), whereas pre-exposure to E2 significantly prevented the reduction in MTT (10%–15% reduction relative



**Figure 3.** Effect of phytoestrogens, genistein (A), genistin (B), daidzein (C), daidzin (D), formononetin (E), and equol (F) on the glutamate-induced neurotoxicity in hippocampal neurons. Primary hippocampal neurons (7 days old) were treated with the phytoestrogens for 48 hr at different concentrations prior to exposure to 100  $\mu$ M glutamate for 10 min. Results are presented as the percentage of control LDH release. Data are expressed as mean  $\pm$  SEM and  $n \geq 6$  per condition. Data are derived from a single experiment and are representative of at least three separate experiments. + $P < 0.001$  compared with control cultures, and \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$  compared with glutamate-treated cultures.

to glutamate-treated cells). In contrast, none of the phytoestrogens was effective in preventing the decline in MTT levels (Table I).

**Neuroprotective Efficacy of Phytoestrogens against  $\beta$ -Amyloid<sub>25-35</sub>.** In addition to excitotoxic glutamate, we analyzed the efficacy of phytoestrogens to protect against  $\beta$ -amyloid<sub>25-35</sub>-induced neurotoxicity. Hippocampal neurons derived from E18-d rat fetuses were cultured in Neurobasal medium for 7 days and treated with test phytoestrogens at 10 ng/ml, 100 ng/ml, and 1  $\mu$ g/ml for 48 hr prior to exposure to 8  $\mu$ g/ml  $\beta$ -amyloid<sub>25-35</sub> peptide for 24 hr. Within the same experiment, 100 ng/ml 17 $\beta$ -estradiol was used as positive control. Exposure to  $\beta$ -amyloid<sub>25-35</sub> alone induced a significant increase in LDH release (ranging from 10% to 25%) compared with control. 17 $\beta$ -estradiol significantly reduced LDH efflux compared with  $\beta$ -amyloid<sub>25-35</sub> alone-treated cultures by 10%–15%. All phytoestrogens tested significantly decreased LDH release induced by  $\beta$ -amyloid<sub>25-35</sub> peptide fragment. Relative to  $\beta$ -amyloid<sub>25-35</sub> alone, genistein, genistin, daidzein, daidzin, formononetin, and equol induced a 5%–15% reduction in LDH release (Fig. 4). Neither 17 $\beta$ -estradiol nor any of the test phytoestrogens alone had effect on LDH release by hippocampal neurons (data not shown).

We also determined the impact of these phytoestrogens on MTT reduction by viable mitochondria in hippocampal neurons following  $\beta$ -amyloid<sub>25-35</sub> peptide exposure.  $\beta$ -amyloid<sub>25-35</sub> peptide alone significantly decreased accumulation of reduced MTT by viable mitochondria relative to control. 17 $\beta$ -Estradiol significantly prevented the MTT decrease. However, similar to the case in glutamate-induced toxicity, no phytoestrogens were effective in preventing the decline in MTT levels induced by  $\beta$ -amyloid<sub>25-35</sub> peptide (Table II).

**Neurotrophic Efficacy of Genistein and Daidzein.** We further investigated the neurotrophic effects of genistein and daidzein on hippocampal neurons. Hippocampal

neurons derived from E18-d rat fetuses were cultured in serum-free Neurobasal media in the absence and presence of genistein or daidzein at concentrations of 1 ng/ml, 10 ng/ml, and 100 ng/ml for 24 hr. Seventy-five neurons per condition were assessed for neurite number, neurite length, branch number, branch length, bifurcation number, and microspike number. Results of these analyses indicated that no significant differences between control neurons and genistein- or daidzein-treated neurons on any of the six morphological features, whereas E2 significantly increased all the parameters (Tables III and IV).

## Discussion

Alzheimer's disease is a neurodegenerative condition characterized by a progressive loss of cognitive capacity manifested as a deterioration of memory function throughout the course of the disease and the development of psychotic symptoms later in the disease process (19, 24). Because women live longer than men and because age is the greatest risk factor for Alzheimer's disease, it can be anticipated that women will have a greater incidence of the disease (19, 24). Although this is indeed true, age is not the only contributing variable because when age is controlled, women are still two to three times more likely to develop Alzheimer's disease when compared with their age-matched male counterparts (47).

The purpose of this study was to determine whether select phytoestrogens, genistein, genistin, daidzein, daidzin, formononetin, and equol could exert either neuroprotective or neurotrophic effects on cultured hippocampal neurons. Results of these analyses indicated that these phytoestrogens exerted a modest but significant reduction in a marker of plasma membrane damage, LDH release, but were ineffective in preventing a decline in neuronal mitochondrial viability following exposure to neurotoxic agents associated with neurodegenerative disease. Furthermore, analyses to

**Table I.** Effect of Phytoestrogens (phyto) versus 17 $\beta$ -Estradiol (E2) on MTT Reduction in Hippocampal Neurons Following Exposure to Excitotoxic Levels of Glutamate

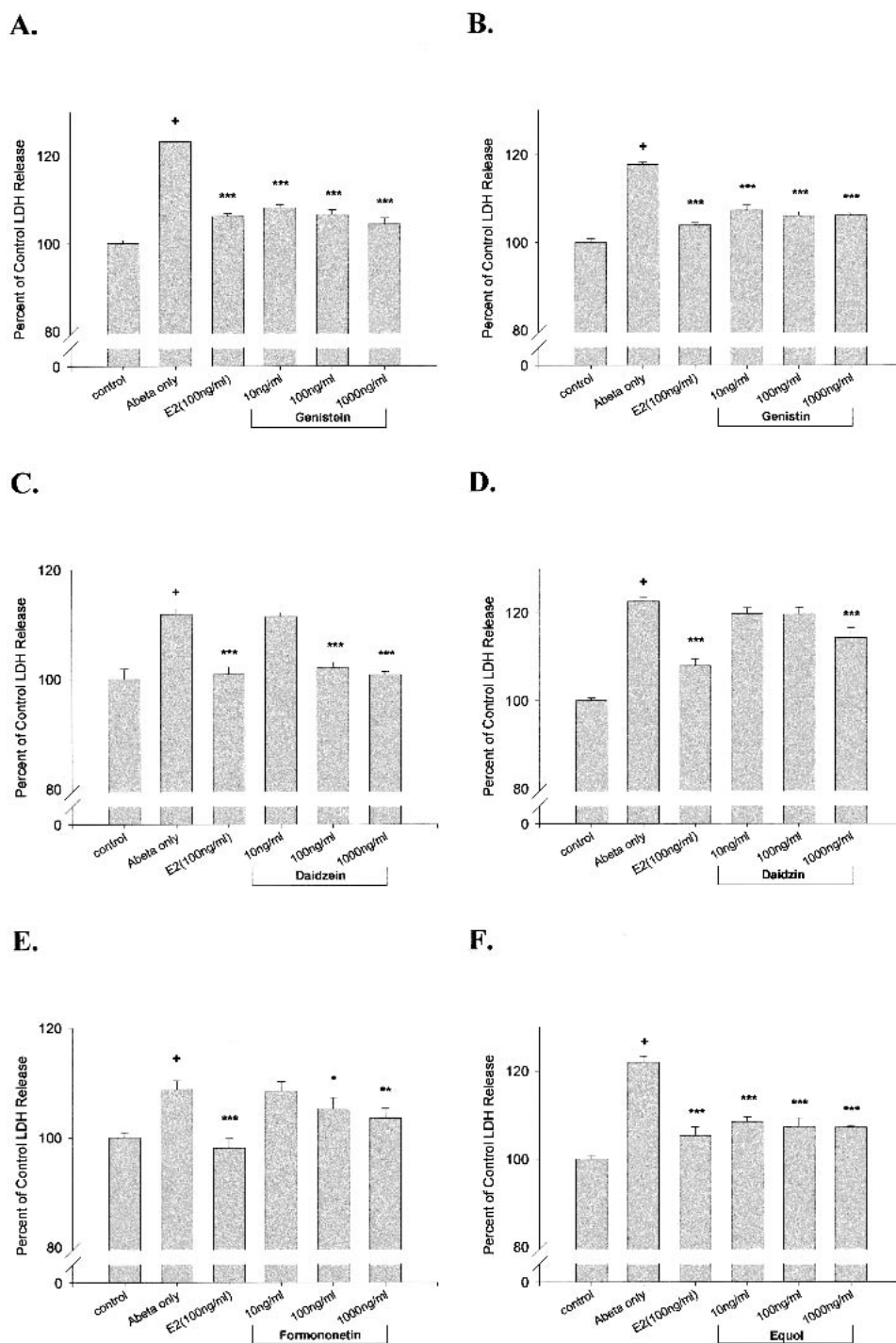
Phytoestrogen added	Control	Glutamate (100 $\mu$ M)	E2 (100 ng/ml) + glutamate	10 ng/ml phyto + glutamate	100 ng/ml phyto + glutamate	1 $\mu$ g/ml phyto + glutamate
Genistein	100 $\pm$ 1	71 $\pm$ 1 <sup>a</sup>	79 $\pm$ 2	72 $\pm$ 2	73 $\pm$ 3	73 $\pm$ 2
Genistin	100 $\pm$ 1	64 $\pm$ 1 <sup>a</sup>	77 $\pm$ 4 <sup>c</sup>	64 $\pm$ 1	68 $\pm$ 1	70 $\pm$ 2
Daidzein	100 $\pm$ 3	71 $\pm$ 2 <sup>a</sup>	81 $\pm$ 2 <sup>b</sup>	70 $\pm$ 1	74 $\pm$ 2	76 $\pm$ 1
Daidzin	100 $\pm$ 2	64 $\pm$ 1 <sup>a</sup>	75 $\pm$ 2 <sup>c</sup>	63 $\pm$ 1	64 $\pm$ 2	67 $\pm$ 3
Formononetin	100 $\pm$ 2	64 $\pm$ 1 <sup>a</sup>	74 $\pm$ 1 <sup>c</sup>	65 $\pm$ 1	68 $\pm$ 2	68 $\pm$ 4
Equol	100 $\pm$ 2	67 $\pm$ 1 <sup>a</sup>	74 $\pm$ 2	66 $\pm$ 1	70 $\pm$ 3	70 $\pm$ 3

*Note.* Primary hippocampal neurons (7 days old) were treated with 17 $\beta$ -estradiol (100 ng/ml) or the phytoestrogens at 10 ng/ml, 100 ng/ml, or 1  $\mu$ g/ml for 2 days prior to 100  $\mu$ M glutamate exposure for 10 min. Results are presented as the percentage of control MTT reduction. Data are expressed as mean  $\pm$  SEM and  $n \geq 6$  per condition. Data are derived from a single experiment and are representative of at least three separate experiments.

<sup>a</sup>  $P < 0.001$  compared with control cultures.

<sup>b</sup>  $P < 0.01$  compared with glutamate-treated cultures.

<sup>c</sup>  $P < 0.001$  compared to glutamate-treated cultures.



**Figure 4.** Effect of phytoestrogens, genistein (A), genistin (B), daidzein (C), daidzin (D), formononetin (E), and equol (F) on the  $\beta$ -amyloid<sub>25-35</sub> peptide-induced neurotoxicity in hippocampal neurons. Primary hippocampal neurons (7 days old) were treated with the phytoestrogens prior to exposure to 8  $\mu$ g/ml  $\beta$ -amyloid<sub>25-35</sub> peptide for 24 hr. Results are presented as the percentage of control LDH release. Data are expressed as mean  $\pm$  SEM and  $n \geq 6$  per condition. Data are derived from a single experiment and are representative of at least three separate experiments. + $P < 0.001$  compared with control cultures, and \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$  compared with glutamate-treated cultures.

determine whether phytoestrogens could exert a neurotrophic effect similar to full estrogen agonists (27, 32, 48) indicated that genistein and daidzein were ineffective as neurotrophic molecules.

Data from a number of laboratories have documented that estrogen exerts a positive effect on specific forms of memory function and that loss of estrogen either through surgical or natural menopause results in a decline in memory function (47–51). Moreover, ERT appears to reduce the risk of developing Alzheimer's disease (19–24). A

number of investigators have found that 17 $\beta$ -estradiol protects neurons against damages induced by  $\beta$ -amyloid, glutamate, and hydrogen peroxide (25–28). Results from our laboratory and others have shown that 17 $\beta$ -estradiol can also significantly increase the outgrowth of neuronal projections and the formation of neural circuits in brain regions critical to memory function (27–34). The inability of phytoestrogens to enhance neuronal survival (evidenced by MTT assays) or to promote neuronal process outgrowth (evidenced by the neurotrophic data) suggests that phytoes-

**Table II.** Effect of Phytoestrogens (phyto) versus 17 $\beta$ -estradiol (E2) on MTT Reduction in Hippocampal Neurons Following Exposure to  $\beta$ -Amyloid<sub>25-35</sub> Peptide

Phytoestrogen added	Control	$\beta$ -amyloid (8 $\mu$ g/ml)	E2 (100 ng/ml) + $\beta$ -amyloid	10 ng/ml phyto + $\beta$ -amyloid	100 ng/ml phyto + $\beta$ -amyloid	1 $\mu$ g/ml phyto + $\beta$ -amyloid
Genistein	100 $\pm$ 2	57 $\pm$ 1 <sup>a</sup>	80 $\pm$ 3 <sup>d</sup>	59 $\pm$ 2	59 $\pm$ 3	61 $\pm$ 2
Genistin	100 $\pm$ 2	63 $\pm$ 1 <sup>a</sup>	67 $\pm$ 1 <sup>c</sup>	59 $\pm$ 2	61 $\pm$ 1	61 $\pm$ 1
Daidzein	100 $\pm$ 2	55 $\pm$ 1 <sup>a</sup>	67 $\pm$ 1 <sup>d</sup>	58 $\pm$ 1	57 $\pm$ 2	59 $\pm$ 1
Daidzin	100 $\pm$ 3	60 $\pm$ 2 <sup>a</sup>	82 $\pm$ 1 <sup>d</sup>	60 $\pm$ 2	60 $\pm$ 2	61 $\pm$ 3
Formononetin	100 $\pm$ 2	59 $\pm$ 1 <sup>a</sup>	73 $\pm$ 2 <sup>b</sup>	64 $\pm$ 4	62 $\pm$ 6	65 $\pm$ 4
Equol	100 $\pm$ 1	66 $\pm$ 1 <sup>a</sup>	79 $\pm$ 1 <sup>d</sup>	70 $\pm$ 5	71 $\pm$ 5 <sup>b</sup>	70 $\pm$ 6

Note. Primary hippocampal neurons (7 days old) were treated with 17 $\beta$ -estradiol (100 ng/ml) or the phytoestrogens at 10 ng/ml, 100 ng/ml, or 1  $\mu$ g/ml for 48 hr prior to exposure to 8  $\mu$ g/ml  $\beta$ -amyloid<sub>25-35</sub> peptide for 24 hr. Results are presented as the percentage of control MTT reduction. Data are expressed as mean  $\pm$  SEM and  $n \geq 6$  per condition. Data are derived from a single experiment and are representative of at least three separate experiments.

<sup>a</sup>  $P < 0.001$  compared with control cultures.

<sup>b</sup>  $P < 0.05$  compared with  $\beta$ -amyloid<sub>25-35</sub>-treated cultures.

<sup>c</sup>  $P < 0.01$  compared with  $\beta$ -amyloid<sub>25-35</sub>-treated cultures.

<sup>d</sup>  $P < 0.001$  compared with  $\beta$ -amyloid<sub>25-35</sub>-treated cultures.

**Table III.** Neurotrophic Efficacy of Genistein in Cultured Hippocampal Neurons

Genistein concentration	Length of neurites ( $\mu$ m)	Number of neurites	Length of branches ( $\mu$ m)	Number of branches	Number of bifurcations	Number of microspikes
0 ng/ml	318.04 $\pm$ 15.79	4.92 $\pm$ 0.23	153.26 $\pm$ 10.74	4.17 $\pm$ 0.32	0.59 $\pm$ 0.10	7.40 $\pm$ 0.34
1 ng/ml	311.47 $\pm$ 19.38	4.99 $\pm$ 0.26	146.81 $\pm$ 13.93	4.29 $\pm$ 0.25	0.63 $\pm$ 0.15	7.33 $\pm$ 0.34
10 ng/ml	321.00 $\pm$ 16.62	4.92 $\pm$ 0.22	149.76 $\pm$ 14.69	3.47 $\pm$ 0.29	0.63 $\pm$ 0.12	4.04 $\pm$ 0.37
100 ng/ml	333.54 $\pm$ 17.63	4.93 $\pm$ 0.22	155.84 $\pm$ 12.89	3.84 $\pm$ 0.29	0.57 $\pm$ 0.10	6.73 $\pm$ 0.31
E2 control (10 ng/ml)	431.10 $\pm$ 33.1 <sup>b</sup>	6.52 $\pm$ 0.50 <sup>b</sup>	256.67 $\pm$ 25.67 <sup>c</sup>	7.76 $\pm$ 0.86 <sup>c</sup>	1.15 $\pm$ 0.13 <sup>c</sup>	16.09 $\pm$ 1.84 <sup>c</sup>

Note. Hippocampal neurons derived from E18-d rat fetuses were cultured in serum-free Neurobasal media in the absence and presence of genistein at concentrations of 1, 10, and 100 ng/ml or 17 $\beta$ -estradiol (E2, 10 ng/ml) for 24 hr. Seventy-five cells per condition were assessed for neurotrophic parameters, including length of neurites, number of neurites, length of branches, number of branches, number of bifurcations, and number of microspikes. Data are derived from a single experiment and are representative of at least three separate experiments. No significant effect on six morphological indicators of neuronal process outgrowth was observed in genistein-treated cells, whereas E2 significantly increased all the parameters.

<sup>b</sup>  $P < 0.01$  compared with control cultures.

<sup>c</sup>  $P < 0.001$  compared with control cultures.

**Table IV.** Neurotrophic Efficacy of Daidzein in Cultured Hippocampal Neurons

Daidzein concentration	Length of neurites ( $\mu$ m)	Number of neurites	Length of branches ( $\mu$ m)	Number of branches	Number of bifurcations	Number of microspikes
0 ng/ml	259.70 $\pm$ 15.04	4.15 $\pm$ 0.21	155.77 $\pm$ 12.12	4.81 $\pm$ 0.36	1.31 $\pm$ 0.18	7.11 $\pm$ 0.35
1 ng/ml	241.85 $\pm$ 15.29	4.01 $\pm$ 0.22	142.64 $\pm$ 13.74	4.69 $\pm$ 0.36	1.18 $\pm$ 0.20	7.25 $\pm$ 0.45
10 ng/ml	254.35 $\pm$ 14.92	4.05 $\pm$ 0.24	166.19 $\pm$ 15.72	5.48 $\pm$ 0.43	1.44 $\pm$ 0.25	7.79 $\pm$ 0.39
100 ng/ml	242.96 $\pm$ 15.90	4.05 $\pm$ 0.19	165.36 $\pm$ 14.93	5.19 $\pm$ 0.42	1.41 $\pm$ 0.21	7.51 $\pm$ 0.46
E2 control (10 ng/ml)	352.02 $\pm$ 27.03 <sup>b</sup>	5.50 $\pm$ 0.44 <sup>b</sup>	260.87 $\pm$ 26.09 <sup>c</sup>	8.95 $\pm$ 0.99 <sup>c</sup>	2.55 $\pm$ 0.28 <sup>c</sup>	15.46 $\pm$ 1.77 <sup>c</sup>

Note. Hippocampal neurons derived from E18-d rat fetuses were cultured in serum-free Neurobasal media in the absence and presence of daidzein at concentrations of 1, 10, and 100 ng/ml or 17 $\beta$ -estradiol (E2, 10 ng/ml) for 24 hr. Seventy-five cells per condition were assessed for neurotrophic parameters, including length of neurites, number of neurites, length of branches, number of branches, number of bifurcations, and number of microspikes. Data are derived from a single experiment and are representative of at least three separate experiments. No significant effect on six morphological indicators of neuronal process outgrowth was observed in daidzein-treated cells, whereas E2 significantly increased all the parameters.

<sup>b</sup>  $P < 0.01$  compared with control cultures.

<sup>c</sup>  $P < 0.001$  compared with control cultures.

trogens are not functional equivalents to endogenously active 17 $\beta$ -estradiol. Therefore, if the neurotrophic effects of 17 $\beta$ -estradiol on the brain mediate memory function, then phytoestrogens would not be expected to sustain memory throughout menopause.

Research over the last 5 years has focused on exploring the intracellular mechanisms of neuroprotective and neurotrophic effects exerted by 17 $\beta$ -estradiol. Several studies have suggested that ER $\alpha$  is involved in 17 $\beta$ -estradiol-induced neuroprotection (52, 53). Neuroprotective effects of

estrogens were abolished in ER $\alpha$ -knockout mice and were intact in ER $\beta$ -knockout mice (54). Phytoestrogens have been found to weakly bind to ER $\alpha$ , whereas they preferentially bind to ER $\beta$  (9). The preferential binding of phytoestrogens to ER $\beta$  may explain why phytoestrogens are not functional equivalents to full estrogen agonists in promoting mechanisms of neuroprotection and neurotrophism. Furthermore, *in vivo*, phytoestrogens may even serve as an antagonist to ERs, thereby blocking ERs and inhibit endogenous estrogen action.

LDH is a stable cytoplasmic enzyme present in all cells and is rapidly released into the cell culture supernatant when the plasma membrane is damaged, thus, it can be used as a reliable biochemical index for neuronal plasma membrane damage. Because extracellular LDH remains stable for days, the magnitude of relative efflux of LDH correlates in linear fashion with the number of damaged neurons in culture (55, 56). MTT is an index of mitochondrial viability because it is reduced by metabolically active cells (57–60). Results of the LDH and MTT analyses indicate that one cannot expect these two cell viability indexes to have reciprocal responses to any chemical agent because they are biochemical markers of different cellular processes. In the case of phytoestrogens, they protected against damage to neuronal membranes, but did not exert a significant effect on neuronal viability as measured by mitochondrial function.

Several mechanisms may underlie phytoestrogen-inducible neuronal membrane protection against toxic insults such as  $\beta$ -amyloid and glutamate toxicity. Phytoestrogens have been suggested to be antioxidants in both chemical and biological model systems (61, 62), which can account for the data that phytoestrogens decreased LDH release induced by the neurotoxic insults of glutamate and  $\beta$ -amyloid. Glutamate and  $\beta$ -amyloid can induce intracellular accumulation of reactive oxygen species to cause cell membrane damage. Phytoestrogens, acting as antioxidants, can decrease the accumulation of reactive oxygen species, thereby protecting cell membrane integrity. Beyond chemical antioxidant properties, other biochemical mechanisms might also play a role. Because phytoestrogens can act as partial estrogen agonists, they could bind to ERs to activate intracellular signaling pathway, leading to kinase activation, transcriptional factor activation, and gene transcription, which are potentially protective of neuronal membrane integrity. Recently, Singh and Toran-Allerand (63) reported that estradiol activated the MAPK signaling pathway in brain. Singer et al. (64) followed up this observation and demonstrated that blockade of this pathway abolished estrogen-induced neuroprotection against glutamate-induced toxicity. Furthermore, Singh (65) recently reported estradiol activation of Akt/protein kinase B, which can mediate the anti-apoptotic signaling pathway through increased expression of the anti-apoptotic protein Bcl-2. Although the above signaling events were shown to be activated by estradiol, the

same mechanisms might be activated by phytoestrogens, mediating their neuronal membrane protective effect. These studies have yet to be conducted. However, the data presented in the current study are also consistent with an antioxidant effect. It is not yet determined whether the neuroprotection conferred by phytoestrogens is mediated by a scavenging antioxidant mechanism or by activation of signaling or genomic cascades.

The data on phytoestrogens and cognition are variable. In animal studies, Clarkson and coworkers (66) found that a soy diet improved working memory in the ovariectomized retired breeder female rats. Furthermore, their results indicated that a soy diet did not antagonize the beneficial effects of estradiol on the working memory of these rats (66). However, these studies did not control for the impact of the nonphytoestrogen component of the diet. Although their findings are encouraging, studies in humans are not consistent with the animal data. Rice and coworkers (67) studied Japanese-American women 65 years or older living in King County, Washington to determine the impact of soy consumption and cognitive function. In women using ERT but who consumed tofu more than three times per week were not protected against cognitive impairment. On the other hand, women using ERT who consumed tofu less than three times per week were less likely to be cognitively impaired than were non-estrogen users. These data suggest that in women already using ERT, consumption of high amounts of tofu can block the beneficial effects of ERT, thereby acting as estrogen antagonists. Furthermore, a recent Honolulu-Asia Aging study by White and colleagues (68) showed a correlation between mid-life consumption of tofu with late life cognitive impairment and dementia. Thus, it would appear that effects of phytoestrogens on cognition are multifaceted, depending on the regime and dose of phytoestrogen consumption.

To date, there is no evidence that a soy-based diet protects against Alzheimer's disease, as Asian countries, such as China and Japan, have rates of Alzheimer's disease similar to rates cited for the United States (69). Suh and colleagues (69) found that overall prevalence rates for Alzheimer's disease and vascular dementia were equal in men, but Alzheimer's disease was predominant in women. In fact, Alzheimer's disease has become nearly twice as prevalent as vascular dementia in Japan, China, and Korea (70).

In conclusion, the present data indicate that although phytoestrogens exhibited a modest degree of neuroprotective efficacy, they did not protect against declines in neuronal viability nor did they induce cellular correlates of memory. These *in vitro* data would predict that individual phytoestrogens are not functional equivalents to estrogen agonist in the brain. Therefore, these data raise the concern that they may not reduce the risk of Alzheimer's disease or sustain memory function in postmenopausal women. A definitive test of this prediction requires a randomized placebo-controlled double-blind clinical trial.

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